Cecil Czerkinsky
Fabienne Anjuere
Jerry R. McGhee
Annie George-Chandy
Jan Holmgren
Marie-Paule Kieny
Kohtaro Fujiyashi
Jiri F. Mestecky
Valérie Pierrefite-Carle
Carola Rask
Jia-Bin Sun

Mucosal immunity and tolerance: relevance to vaccine development

Authors' addresses
Cecil Czerkinsky[1], Fabienne Anjuere[1], Jerry R. McGhee[2], Annie George-Chandy[1,3], Jan Holmgren[3], Marie-Paule Kieny[4], Kohtaro Fujiyashi[2], Jiri F. Mestecky[2], Valérie Pierrefite-Carle[1], Carola Rask[3], Jia-Bin Sun[3],
[1]INSERM Unit 364, Faculté de Médecine-Pasteur, Nice, France.
[2]Department of Microbiology and The Immunobiology Vaccine Center, The University of Alabama at Birmingham, Birmingham, Alabama, USA.
[3]Department of Medical Microbiology and Immunology, University of Göteborg, Göteborg, Sweden.
[4]INSERM Unit 74, Institute of Virology, Strasbourg, France.

Correspondence to:
Cecil Czerkinsky
INSERM Unit 364
Faculté de Médecine-Pasteur
Nice
France
Fax: 33 493 81 9445
e-mail: czerkinsky@dial-up.com

Acknowledgements
Studies from the authors laboratories are supported by INSERM (France), the European Communities (Biotechnology and Biomedical Programs), the Swedish Medical Research Council, and the U. S. National Institutes of Health.

Immunological Reviews 1999
Vol. 170: 197–222

Immunological Reviews
ISSN 0105-2896

Summary: The mucosal immune system of mammals consists of an integrated network of lymphoid cells which work in concert with innate host factors to promote host defense. Major mucosal effector immune mechanisms include secretory antibodies, largely of immunoglobulin A (IgA) isotype, cytotoxic T cells, as well as cytokines, chemokines and their receptors. Immunologic unresponsiveness (tolerance) is a key feature of the mucosal immune system, and deliberate vaccination or natural immunization by a mucosal route can effectively induce immune suppression. The diverse compartments located in the aerodigestive and genitourinary tracts and exocrine glands communicate via preferential homing of lymphocytes and antigen-presenting cells. Mucosal administration of antigens may result in the concomitant expression of secretory immunoglobulin A (S-IgA) antibody responses in various mucosal tissues and secretions, and under certain conditions, in the suppression of immune responses. Thus, developing formulations based on efficient delivery of selected antigens/tolerogens, cytokines and adjuvants may impact on the design of future vaccines and of specific immunotherapeutic approaches against diseases associated with untoward immune responses, such as autoimmune disorders, allergic reactions, and tissue-damaging inflammatory reactions triggered by persistent microorganisms.

Introduction

The mucous membranes covering the aerodigestive and the urogenital tracts as well as the eye conjunctiva, the inner ear and the ducts of all exocrine glands are endowed with powerful mechanical and physicochemical cleansing mechanisms that degrade and repel most foreign matter. In addition, a large and highly specialized immune system protects these surfaces and thereby also the body interior against potential insults from the environment. In a healthy human adult, this local immune system contributes almost 80% of all immunocytes. These cells are accumulated in or in transit between various mucosal organs and glands and together they form the mucosa-associated lymphoid tissue (MALT), the largest mammalian lymphoid organ system.

The MALT has three main functions: 1) to protect the mucous membranes against colonization and invasion by potentially dangerous microbes encountered; 2) to prevent

uptake of undegraded antigens including foreign proteins derived from ingested food and commensal microorganisms; and 3) to prevent the development of harmful immune responses to these antigens if they do reach the body interior. At variance with the systemic immune apparatus, which functions in a normally sterile milieu and often responds vigorously to invaders, the MALT guards organs that are replete with foreign matter. It follows that upon encounter with this plethora of antigenic stimuli, the MALT must ignore such antigens, or economically select appropriate effector mechanisms and regulate their intensity to avoid bystander tissue damage and immunological exhaustion. Since this system cannot discriminate between pathogenic microorganisms on the one hand, and innocuous matter, such as dietary matter and commensal microorganisms, on the other, it is likely to recognize all foreign peptides. Elucidating the mechanisms that determine whether recognition of such diverse antigens will result in active immunity, immune suppression or ignorance, will have a major impact in the development of effective mucosal vaccines against infectious and inflammatory diseases.

Although the immune apparatus is remarkably diverse, there is strong evidence that certain types of immune responses take place and are basically restricted to certain anatomic locations within the body. The MALT represents a well-known example of such a compartmentalized immunological system. As opposed to the central and peripheral lymphoid organs, the MALT contains inhomogeneously distributed B and T cells whose phenotype, repertoire, developmental origin, secretion products and hence probably also function are different. First, the prime immunoglobulin isotype produced and assembled in mucosal tissues is secretory immunoglobulin A (IgA), which is only present in trace amounts in the intravascular compartment. Further, and although major species differences have been observed, non-conventional lymphocytes rarely seen in the spleen and peripheral lymph nodes are encountered at appreciable frequency in different mucosal locations, e.g. α/β $TCR^+CD4^-CD8^-$ cells, γ/δ TCR^+CD4^-CD8 α^+CD8 β^- and γ/δ TCR^+CD4^-CD8 α^-CD8 β^- T cells in the gut epithelium. The antigen receptor repertoire is also different in each location. Thus, self-reactive T cells are detected among murine intestinal intraepithelial α/β and γ/δ TCR^+ T lymphocytes. Furthermore, in contrast to the T and B lymphocytes found in central and peripheral lymphoid organs, certain γ/δ and α/β T cells found in the mucosa, and presumably also mucosal $CD5^+$ B cells, do not depend on the thymus or bone marrow for their development, respectively. In addition to divergent lineages that are driven to different organs by tissue-specific homing receptors, local selection mechanisms may be important in the diversification of mucosal immune responses. Selection may be exerted by local antigens, antigen-presenting cells (APCs) whose function varies in each anatomical location, cytokines, chemokines and cell-matrix interactions, thus leading to the expansion and maintenance of some clones, whereas others are diluted out or deleted. Thus, by its cellular composition, and through the compartmentalization of its afferent and efferent limbs, the MALT functions essentially independently of the systemic immune apparatus.

Although most infectious microorganisms colonize or enter the host through mucosal membranes, it is the systemic immune system which has been the focus of most vaccine research. Aside from the ease of their administration, an important advantage of mucosal vaccines is the theoretical possibility of inducing both mucosal and systemic immune responses, while the reverse does not hold true. Since specific humoral immune defense is provided by both serum and by secretory antibodies, predominately of the IgA isotype, that are selectively transported into external secretions, most vaccines considered for further improvement or development should ideally induce both systemic and mucosal responses.

Equally important to host immune defense is the capacity of MALT to promote specific immunological unresponsiveness (tolerance) after natural or deliberate mucosal exposure to a variety of antigens. This form of tolerance is considered as a major adaptive immune defense mechanism whereby we avoid developing harmful immune responses against the plethora of dietary and airborne antigens encountered each day.

Thus, from a theoretical standpoint, the possibility of manipulating the mucosal immune system towards positive immunity and/or tolerance appears extremely attractive when considering strategies aimed at protecting the host from colonization or invasion by microbial pathogens but also to prevent and/or to modulate the development of potentially harmful immunological reactions against the same pathogens and also against certain self antigens and allergens. Such considerations should finally bring mucosal vaccines to center stage in vaccine development.

Adaptive effector mechanisms in mucosal immune defense

Three major adaptive effector mechanisms participate in the immune defense of mucosal surfaces. Secretory antibody formation and antigen-specific cell-mediated cytotoxicity are the primary mechanisms involved in antimicrobial defense and act primarily but not exclusively in the epithelium of all mucosal tissues. In addition, a third form of mucosal immune defense is contributed by regulatory cells which act in both epithelium

and submucosa as well as in extramucosal tissues mainly through the production of soluble mediators. Although these cells may regulate IgA antibody formation and the development of cell-mediated cytotoxic responses, they participate in the maintainance of mucosal tolerance against most environmental matters and as such can be regarded as key players in mucosal defense against inflammation.

Secretory IgA

Immune responses expressed in mucosal tissues are typified by secretory immunoglobulin A (S-IgA) antibodies. S-IgA constitutes the predominant Ig class in human external secretions, and is the best known entity providing specific immune protection for mucosal tissues. While the synthesis and assembly of J chain-containing polymeric IgA molecules is contributed to by plasma cells, mucosal epithelial cells synthesize a secretory component which acts as a cell surface receptor to facilitate the transport of newly formed IgA across epithelial cells. The resistance of S-IgA against endogenous proteases makes antibodies of that isotype uniquely well suited to protect mucosal surfaces. S-IgA antibodies provide "immune exclusion" of bacterial and viral pathogens, bacterial toxins and other potentially harmful molecules, a function that appears to be facilitated by its affinity for binding to mucus. S-IgA has also been reported to neutralize directly a number of viruses (1, 2), to mediate antibody-dependent cell-mediated cytotoxicity, and to interfere with the utilization of growth factors for bacterial pathogens in the mucosal environment.

Mucosally derived S-IgA differs from bone marrow-derived serum IgA, not only in terms of specific antibody activity but also in the proportions of polymeric vs monomeric forms (3). The ontogenies of the mucosal and systemic IgA compartments display characteristic and apparently independent patterns of maturation. Adult levels of S-IgA are reached in external secretions considerably earlier (1 month to 2 years) than in the serum (adolescence) (4, 5). Experiments that addressed the origin of mucosal antibodies have indicated that an overwhelming proportion of such antibodies are produced locally in mucosal tissues and that only a minor fraction derive from the circulation in most species, including humans (6, 7).

Mucosal cytotoxic T lymphocytes

Although the focus of most investigations has been on mucosal cytotoxic T lymphocytes (CTLs) induced by viruses, it should be borne in mind that antibody-mediated cytotoxicity and natural killer (NK)-cell activity are major responses associated with mucosal lymphocytes (8–14). The relevance of the latter responses in the field of mucosal vaccine development has not been ascertained and is therefore beyond the scope of this review.

Two major effector mechanisms associated with CTLs have been described: exocytosis from CTLs onto target cells of granules containing the pore-forming protein perforin and several serine proteases also termed granzymes, and ligation of a tumor necrosis factor (TNF) receptor-like molecule (Fas or CD95) on the target cell by FasL or TNF on the CTL side (15).

CTLs in the gut mucosa

An early sudy demonstrated that intraperitoneal immunization with an allogenic tumour induced specific CTLs in the gut lamina propria and epithelium at a time when very few cytotoxic lymphocytes could be detected in peripheral lymphoid organs (9).

It is now established that administration of certain enteric viruses into the gastrointestinal tract also results in early appearence of virus-specific CTLs in Peyer's patches (16–21). Further, reovirus infection also induces effector $CD8^+$ $\alpha\beta$ TCR^+ CTLs in the intestinal epithelium (20). Similarly, rotavirus infection induces increased pCTLs in GALT and their dissemination throughout the murine lymphoid system (17). These findings suggest that after enteric infection or immunization, antigen-induced CTLs are disseminated from Peyer's patches via the lymphatic drainage (21), and could serve as one of the sources of pCTL progenitors destined to the epithelial defense system. The potential of this system in immune clearance of enteric viruses has been documented in several systems. Thus, mice orally infected with *Toxoplasma gondii* develop intraepithelial $CD8^+$ $\alpha\beta$ TCR^+ CTLs which can transfer protection when infused into naive recipients (22). Further, effector CTLs protected against gastritis in a suckling mouse model of rotavirus infection (23). In a series of elegant studies designed to define the host determinants of rotavirus immunity, it was shown that adoptively transferred $CD8^+$ T cells mediated clearance of rotavirus infection in severe combined immunodeficiency (SCID) mice (24–26). Although S-IgA can also neutralize rotavirus infection of epithelial enterocytes (27), these studies indicate that $CD8^+$ CTLs are of central importance in rotavirus immunity.

Worth mentioning are the results of recent studies showing that intrarectal immunization of mice with a synthetic, multideterminant human immunodeficiency virus (HIV) peptide induced long-lasting, antigen-specific CTL memory in both the inductive (Peyer's patch) and effector (lamina propria) mucosal sites, and protected mice against infection via mucosal challenge with a recombinant vaccinia virus expressing HIV-1 gp160 (28). These studies provide evidence for the ability of

CTL in the mucosa to mediate protection against viral transmission.

CTLs in the airway mucosa

The kinetics of activated CTLs following virus clearance suggest that these cells do not play a very critical role in prevention of reinfection by respiratory viruses, such as respiratory syncytial virus (RSV), influenza and parainfluenza viruses. In contrast, studies in immunosuppressed animals indicate that CTLs play a central role in the resolution of established respiratory viral infections (29–31). It is likely that CTLs play also a major role in the clearance of established respiratory virus infections in humans. Studies in patients who had undergone immunosuppressive chemotherapy indicate that these patients suffer more frequent and severe infections with RSV, influenza, or parainfluenza viruses (32).

Detailed studies of immune responses after intranasal infection of rodents with influenza virus have revealed additional immune pathways involved in virus clearance. In this model, use of CD4 co-receptor knockouts or other mice in whom this subset had been depleted did not affect induction of pCTLs or alter significantly clearance of infection (33). In another study, clearance of influenza was not altered by the use of β_2 microglobulin knockout mice which lack $CD8^+$ T cells or mice which had been treated with monoclonal antibody (mAb) anti-CD8 (34). In the same model, the finding that γ/δ T cells with several Vδ chain specificities increase in the infected site as clearance occurs raises intriguing questions regarding the regulatory role of γ/δ T cells in antiviral immunity (35).

Several studies have also established that effector CTLs protect mice from RSV infection. The RSV F determinant, a 22 kD glycoprotein, is a major target of pCTLs and CTL induction by RSV or recombinant vaccinia virus expressing F glycoprotein-induced protective CTLs (36, 37). In a separate line of investigation, the murine RSV model was used to determine the relative importance of $CD4^+$ T cells, including T helper 1 (Th1)- and T helper 2 (Th2)-subsets which resulted in inflammation vs immunity. These studies clearly suggest that interferon-γ (IFN-γ)-producing $CD4^+$ Th1 cells as well as $CD8^+$ T cells are associated with recovery, while $CD4^+$ Th2-cells are not (38, 39). Interestingly, priming with inactivated RSV or F glycoprotein induced $CD4^+$ Th2 cells while live RSV elicited the Th1-type pathway (38, 39).

CTLs in the genital tract mucosa

The rhesus macaque model of vaginal infection with simian immunodeficiency virus (SIV) has been useful in studies of local immunity to SIV in the female reproductive tract (40). Recent studies in this model have provided direct evidence that pCTLs occur in female macaque reproductive tissues, and that infection with SIV induces effector CTL responses in the vaginal mucosa (41). Interestingly, macaques vaginally infected with an SIV/HIV-1 chimeric virus (SHIV) displayed gag-specific CTLs in peripheral blood and resisted two challenges with virulent SIV (42).

In a recent study involving HIV-1 infected women, cervical T-cell lines established from cytobrush specimens were shown to lyse autologous targets expressing HIV-1 proteins (43). Class II MHC-restricted $CD4^+$ CTL clones lysed targets expressing env gp41 or infected with HIV-1. Class I major histocompatibility complex (MHC)-restricted $CD8^+$ clones recognized HIV-1 Gag- or Pol-expressing targets. This study provided evidence for an MHC-restricted CTL effector function in the human female genital tract mucosa.

Regulatory T cells in the mucosal immune system

T cells are required for mucosal immunity, whether it develops as inflammation, or as tolerance or as help for specific S-IgA antibodies or CTLs in response to vaccines. B-cell commitment (μ→α switching) and B–T interactions which result in the induction of plasma cells producing polymeric IgA (pIgA) are of central importance to mucosal immunity. Cytokines and chemokines produced by $CD4^+$ and $CD8^+$ T-cell subsets and by classical APCs (e.g. dendritic cells (DCs, macrophages and B cells)) as well as by non-classical APCs (e.g. epithelial cells) contribute to all aspects of normal mucosal immunity, tolerance and inflammation in the immune response.

Regulatory T cells can be classified as: 1) naive, or those which have not yet encountered antigen; 2) activated (effector); and 3) memory types. Effector and memory T cells are both actively engaged in the immune response. The mucosal migration patterns of the three major subsets, along with the homing of B lymphocytes, form the cellular basis for the common mucosal immune system (44). Naive $CD4^+$ precursors of Th cells (pTh) normally recognize foreign peptide in association with MHC class II on APCs and express an αβ TCR^+, $CD3^+$, $CD4^+$, CD8 phenotype. On the other hand, precursor CTLs (pCTLs) express αβ TCR which usually recognize foreign peptide in the context of MHC class I on target cells and exhibit a phenotype of $CD3^+$, $CD4^-$, $CD8^+$. Thus, the MALT can be considered as a significant reservoir of pTh cells and pCTLs so that encounter with bacterial or viral pathogens can result in the induction of $CD4^+$ Th cell and $CD8^+$ CTL responses.

As $CD4^+$ Th cells differentiate in response to foreign antigens, they produce distinct cytokine arrays. Naive (pTh) cells first produce interleukin (IL)-2 and then develop into T cells

producing multiple cytokines (including both IFN-γ and IL-4), a stage often termed Th0 (45, 46). The tissue microenvironment (47), the cytokine milieu and the nature of the antigen influence the further differentiation of Th0 cells. Thus, infection with intracellular bacteria leads to the formation of Th1 cells and these cells often develop following production of IL-12 by activated macrophages (48, 49). Exogenous antigens in the mucosal microenvironment can trigger $CD4^+$, $NK1.1^+$ T cells to produce IL-4 for initiation of Th2-type responses (from Th0 cells) (50, 51). Th2-type cells also produce IL-4 for expansion of the Th2 subset as well as IL-5, IL-6, IL-9, IL-10, and IL-13 (52–56). This Th2 array may include production of IL-4; however, the reader should appreciate that individual cytokines are regulated through different signal transduction pathways so that all Th1 or Th2 cells do not produce the entire array. The Th2 array of cytokines is of importance for B-cell isotype switches, and for supporting IgG1, IgG2b, IgE, and IgA responses in the mouse system (54, 57, 58).

Th1 and Th2 cells are also sensitive to cross-regulation by each other. For example, IL-12 and IFN-γ produced by Th1 cells inhibits proliferation of Th2 cells, causes an isotype switch from IgM to IgG2a (50), and inhibits isotype switching induced by IL-4 (59). Conversely, Th2 cells regulate the effects of Th1 cells by secreting IL-10 which in turn inhibits Th1 cells, from secreting cytokines such as IL-12 and IFN-γ, thus decreasing IFN-γ-mediated inhibition of Th2 cells.

While the bulk of available evidence in mice suggests that mucosal tissues favor the development of Th2-type responses, the situation is not as clear-cut in humans. Thus, freshly isolated human lamina propria T cells contain an exceptionally high frequency of IFN-γ-producing cells (60, 61) but comparatively few cells producing IL-4, IL-5 or IL-10 (61). Furthermore, even in mice, infection with recombinant *Salmonella* leads to the formation of Th1 cells and Th2 cells in Peyer's patches (62). On the other hand, induction of IgA production to protein antigens is highly dependent on T helper cells (63–71). In humans, TGF-β and IL-10 in concert with IL-4 have been shown to promote B-cell differentiation into IgA-producing cells (64, 65). In this regard, it appears that in addition to resident T cells, which produce large amounts of IL-4, IL-10 and transforming growth factor (TGF)-β, human epithelial cells, also of intestinal origin, provide a major source of TGF-β and IL-10 (unpublished observations). This suggests that co-operation between neighboring lymphocytes and epithelial cells in the mucosal microenvironment is pivotal not only to promote the selective transport of newly formed IgA across epithelia but also for programing preferential maturation of IgA-committed B cells.

Finally, an additional type of $CD4^+$ regulatory T cell has been cloned from the mesenteric lymph nodes of rodents fed repeatedly with antigens (72). These cells or "Th3" cells, produce TGF-β, variable amounts of IL-4 and IL-10, but no IL-2 or IFN-γ, and appear to exert downregulatory properties on Th1 cells (73). Since TGF-β happens to serve as switch factor for murine and human IgA, and appears to play a major role in mucosal tolerance (reviewed in (74)), these cells may have an important function in mucosal homeostasis.

Mucosal inductive sites and generation of disseminated and compartmentalized secretory immune responses

Generation of an immune response at mucosal surfaces, where many significant infections begin, is not readily achieved by the conventional route of parenteral injection, although this is usually effective in eliciting circulating antibodies and systemic cell-mediated immune responses. In contrast, mucosal administration of antigens may result in the concomitant expression of antibody responses in various mucosal tissues and secretions, usually without a pronounced systemic immune response (85).

Extensive studies concerning the origin of B- and T-lineage lymphocytes that ultimately populate mucosal tissues and secretory glands, and of immunization routes effective in the induction of secretory antibody responses, indicated that the MALT can be divided into two functionally distinct compartments; namely, inductive and effector sites. This physiological division is of paramount importance in the design of vaccines effective in the induction of protective immunity within the mucosal immune system and, in particular, its humoral branch.

Experiments performed in animal models and more recently in SCID mice engrafted with human mucosal lymphoid cells revealed that the inductive sites present in certain locations, such as Peyer's patches in the small intestine, or in some species the tonsils in the upper aerodigestive tract, function as primary sources of precursor cells that migrate through the lymphatics and blood, and after directed extravasation populate remote mucosal tissues and glands (75–78). These studies have led to the notion of a common mucosal immune system (*Fig. 1*).

It is generally believed that antigens taken up by specialized epithelial cells ("M" cells or "membranous cells") covering mucosal inductive sites can be channeled to parenchymal macrophages, DCs, B lymphocytes, and even mast cells, and/or they can be processed and perhaps presented directly by epithelial cells to underlying B and T cells. Following interaction of the antigen with accessory cells and cognate helper T cells

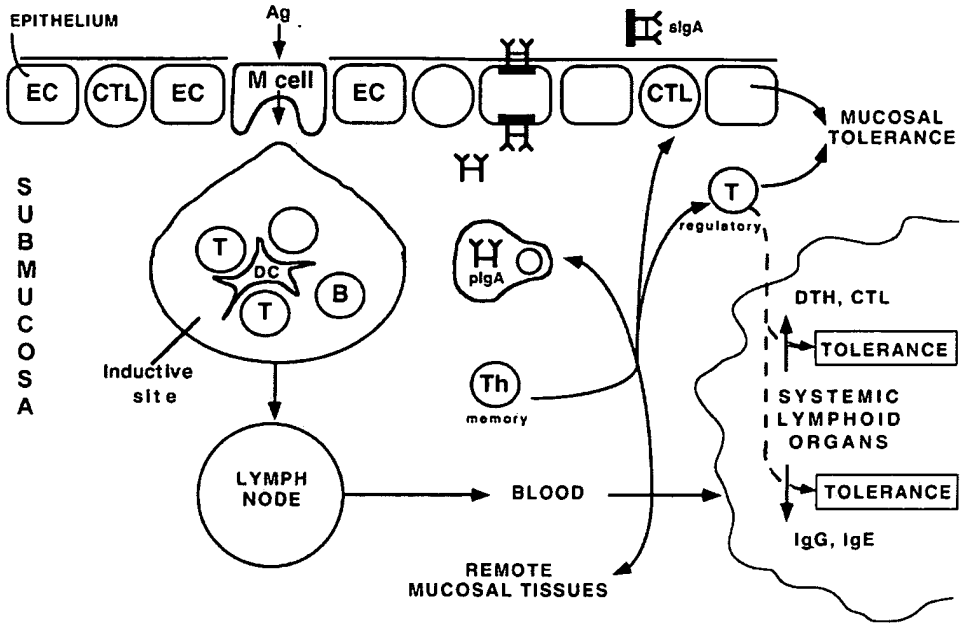

Fig. 1. Consequences of antigen uptake at mucosal surfaces. Uptake of antigen (Ag) through M cells or absorptive epithelial cells (EC) may result in induction of localized or disseminated mucosal and systemic immune responses and/or tolerance. CTL, cytolytic T lymphocytes; DC, dendritic cells; DTH, delayed-type hypersensitivity; pIgA, polymeric IgA; sIgA, secretory IgA.

and/or B lymphocytes in the local lymphoid microenvironment, an immune response may ensue. With the majority of antigens this results presumably in suppression of specific immunity – "mucosal tolerance" (see below). However, development of an active immune response may also follow mucosal intake of antigen, and the characteristics of the immune response generated, including the balance between active immunity and suppression/tolerance may be influenced by several factors, including the nature of the antigen, the type of accessory cells, cytokines and lymphocytes involved, and the genetic background of the host. Two major types of active immune responses may develop either concomitantly or separately: antibody formation (mainly SIgA) and cell-mediated immunity. The sensitized immunocytes, in particular antigen-sensitized B cells but presumably also T cells, leave the site of initial encounter with antigen, e.g. a Peyer's patch, transit through the thoracic duct, enter the circulation and then seed both that same mucosa and other distant yet privileged mucosal sites. In their new locations, the committed B cells may further differentiate into plasma cells producing antibodies, mainly dimeric IgA, under the influence of locally produced cytokines.

This IgA cell cycle was first elucidated in animals through the adoptive transfer of cells from the gut- and bronchus-associated lymphoid tissues (GALT and BALT) into recipients whose mucosal tissues and glands were populated by IgA plasma cells of donor origin. Most importantly for vaccine development, evidence for the existence of a common mucosal immune system in humans has been strengthened in recent years by several studies. In addition to the detection of specific S-IgA antibodies in remote secretions induced by natural exposure to antigens or oral immunization, analyses of IgA-secreting cells from peripheral blood and mucosal tissues after enteric immunization provided strong support for this concept (79–81). Differential utilization of organ-specific endothelial cell recognition mechanisms by circulating IgA immunoblasts induced after mucosal vaccination has also been demonstrated in humans (82, 83). Taken together with studies in the murine

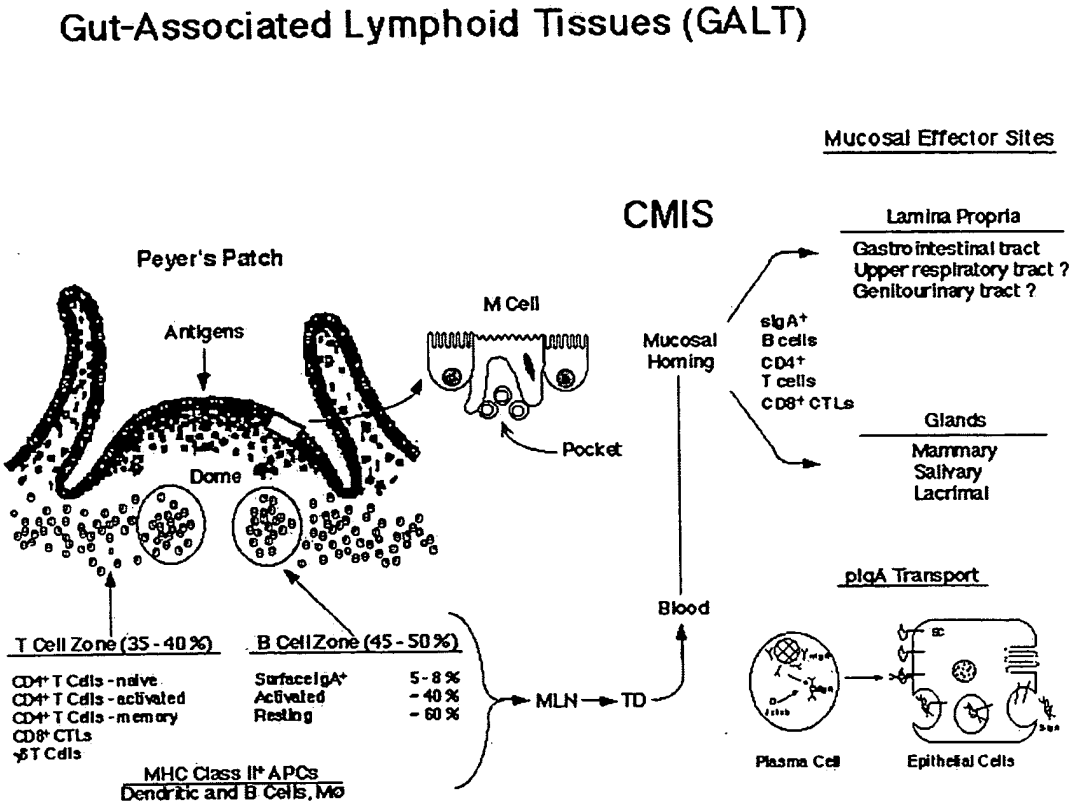

Fig. 2. Gut-associated lymphoid tissues (GALT) and the common mucosal immune system (CMIS). MLN, mesenterial lymph node; TD, thoracic duct; M cell, membranous cell; pIgA, polymeric IgA.

system, this finding may explain both the unification of humoral immune responses in diverse mucosal sites and the physiologic segregation of mucosal from non-mucosal immune mechanisms.

Based on the concept of a common mucosal immune system through which a fraction of lymphocytes activated in the gut, e.g. by ingestion of antigen, can disseminate immunity not only in the intestine but also to other mucosal and glandular tissues, there is currently much interest in the possibility of developing oral vaccines against both enteric and non-enteric mucosal infections, e.g. in the respiratory or genital tract. However, recent studies involving immunizations of human and non-human primates as well as rodents with prototype non-replicating immunogens, such as cholera toxoids or cholera toxin indicate that a substantial degree of subcompartmentalization exists within the mucosal immune system, and even within a given mucosal organ such as the gut, regarding both homing of IgA plasma cell precursors and final redistribution of their progenitors.

The MALT is comprised of anatomically defined lymphoid microcompartments, such as the Peyer's patches in the small intestine, the appendix and solitary follicles in the large intestine and in the rectum, the nasal mucosa and the tonsils at the entrance of the aerodigestive tract, which serve as the principal mucosal inductive sites where immune responses are being initiated (84–87). It also contains diffuse accumulations of large numbers of lymphoid cells that do not associate into apparently organized structures. These cells are either distributed in the lamina propria or interspersed among epithelial cells in mucosal tissues and glands, and form the mucosal effector sites where immune responses are being expressed. The gut mucosa is particularly well invested with such diffuse lymphoid tissues.

More important for vaccine development is the fact that immunization at certain inductive sites may give rise to a humoral immune response preferentially manifested at certain effector sites. Thus, a given inductive site may serve as a preferential but not exclusive source of precursor cells for certain mucosal tissues.

Inductive sites in the gut-associated lymphoid tissues (GALT)

The gut contains the most abundant lymphoid tissues and includes organized as well as diffuse lymphoid elements. Organized lymphoid tissues are comprised of two units – B-cell follicles and para- or interfollicular T-cell areas – assembled within a matrix of loose connective tissue and follicular DCs. These follicles occur singly or in groups and harbour variable numbers of macrophages and T cells (88).

Follicle-dome structures form the main lymphoid component of the Peyer's patches in the jejunum and ileum and are also found in the large intestine, and especially in the appendix. These structures appear to play an important role in the induction of disseminated immune responses to oral vaccines (Fig. 2). Typically, the follicles contain a majority of B cells, approximately half of which are activated. The T-cell zone comprises a majority of $CD4^+$ T cells; $CD8^+$ $\alpha\beta$ TCR T cells are mainly located in the parafollicular area whereas $CD8^+$ $\gamma\delta$ TCR T cells are rare. The dome is covered by a specialized epithelium or "follicle-associated epithelium" (FAE), containing antigen-transporting M cells.

The cecal and colonic mucosae are also variably invested with comparable lymphoid patches, although the epithelium covering these lymphoid aggregates does not show the specializations of M-cell-containing FAEs (89, 90).

Clusters of follicles are also found adjacent to the ano-rectal junction (91, 92). The potential importance of the rectal lymphoid tissues as an IgA inductive site and as a source of IgA plasma cell precursors is suggested by several studies. First, the predominance of IgA2 cells over IgA1 cells in the lamina propria of the large intestine clearly diverges from the relative apportioning of the two in other mucosal tissues, such as in the small intestine and in the upper large intestine (93). Further, rectal immunization of humans, non-human primates and rodents has been shown to induce strong secretory antibody responses in the rectal mucosa. Although in most instances, rectal immunization induced secretory antibody responses in the rectal mucosa itself (94–100), in some instances, rectal vaccination could induce specific antibodies in serum, and also in secretions from remote mucosal organs, such as saliva (102), and genital secretions (92–94, 96). Although the potential of the rectal mucosa to serve as site of induction of mucosal responses is now well established, it should be pointed out that rectal immunization of macaques with CT, one of the most powerful mucosal immunogens, was shown to be poorly effective at inducing an immune response in the upper part of the intestine and especially in the small intestine (100).

Recently, a new type of "organized" lymphoid tissue, termed the cryptopatch, has been identified in the murine intestine (101). These consist of microscopic aggregates (a few hundred cells) of immature lymphocytes clustered in the lamina propria. Although cryptopatches have not yet been identified in humans, it has been proposed that they represent a precursor source of intestinal intraepithelial T cells. The potential role of these structures in the induction of active immunity or tolerance to mucosally encountered antigens remains to be explored.

Inductive sites in the naso-pharyngeal lymphoreticular tissues (NALT)

Several recent studies have emphasized the importance of the nasal cavity for the generation of mucosal and systemic immune responses that may exceed in magnitude those induced by oral immunization (83, 102–111). When introduced into the nasal cavity, usually along with mucosal adjuvants, viral and bacterial antigens induce superior immune responses in external secretions such as saliva and, surprisingly, in female genital tract secretions. Because neither IgG nor IgA antibody titers in vaginal washes correlate with serum antibody responses, it is assumed that antibodies of both isotypes are predominantly of mucosal origin. This finding may have important implications for the design of vaccines effective in the induction of immune responses in the genital tract.

Thus, different immunization routes (intranasal and oral) can induce generalized mucosal immune responses, although the relative representation of dominant antibody isotypes may vary. Nevertheless, nasal immunization appears to induce S-IgA immunity in a broader range of mucosal tissues than oral vaccination. This may be explained by the recent observation that circulating IgA-secreting cells induced after nasal vaccination express a more promiscuous profile of homing receptors than their corresponding counterparts raised after oral or rectal immunization (82, 83). Whether such antibody responses are also induced in the male genital tract remains to be determined.

Immunohistochemical studies have indicated that the epithelium of the human nasal mucosa contains intraepithelial lymphocytes (IELs) staining predominantly for CD3, CD2, CD8, and CD5, but lacking Leu8 (homing receptor analogue of Mel14) (123, 124). Few (<10%) nasal IELs stain for CD4. Nasal IELs rarely express HML1, whereas the majority of intestinal, tonsillar and adenoidal IELs are $HML1^+$. $HLA\text{-}DR^+$ intraepithelial DCs cells have also been identified in nasal mucosa specimens. Virtually no B cells and no organized subepithelial lymphoid tissue are found in the normal human nasal mucosa. In contrast, the nasal mucosa of adult rats and mice comprise lymphoid structures with distinct T- and B-cell areas (112, 113). Furthermore, the majority of intraepithelial lym-

phocytes populating the nasal epithelium are sIg$^+$ B cells with a few CD4$^+$ or CD8$^+$ T cells in rats (112), whereas the murine nasal epithelium is infiltrated mainly with CD4$^+$ T lymphocytes with very few B cells (113). Thus, the human nasal mucosa seems to differ from that of rodents – the most commonly used animals in preclinical vaccine studies – and also from endodermally derived human mucosae, such as the tonsils and adenoids. The latter lymphoid structures appear to be the major sites of induction of mucosal immune responses to inhaled antigens in humans.

Strategically positioned at the entry of the aerodigestive tract, the palatine, lingual and nasopharyngeal tonsils (Waldeyer's ring) are continuously exposed to ingested and inhaled antigens. These glands possess structural features resembling both lymph nodes and Peyer's patches (87), including a lymphoepithelium which contains M cells in tonsillar crypts, and which are essential for antigen uptake (Fig. 2). In addition, germinal centers containing B and T cells, plasma cells, and professional APCs are also present. Several observations have suggested that these lymphoid tissues may serve as a source of precursors of IgA plasma cells found in the upper aerodigestive tracts: the distribution of IgA1- and IgA2-producing cells in the nasal and gastric mucosae and in lacrymal and salivary glands is similar to that found in tonsils. Furthermore, the S-IgA immune response to oral poliovirus vaccine is reduced in tonsillectomized children compared to children with intact tonsils (114). Direct unilateral injection of antigens into the tonsil of human volunteers resulted in the induction of local immune responses manifested by the appearance of antigen-specific IgG-producing and to a lesser degree IgA-producing cells in the injected tonsil (103). Furthermore, considerable numbers of antibody-forming cells with a similar distribution of isotypes were detected in peripheral blood, suggesting that the tonsils may serve as an inductive site, analogous to Peyer's patches, that is effective in the stimulation of generalized mucosal immune responses.

Inductive sites in the genital tract mucosa

Although long considered as immunologically incapable of supporting an active immune response against locally encountered antigens, but especially against spermatozoa or preimplantation embryo, the female reproductive tract mucosa has been shown to comprise all cell populations required for initiating an immune response. HLA-DR$^+$ Langerhans cells have been identified in the vaginal and cervical epithelia (117, 118), being most abundant in the vulval epithelium. Intraepithelial T cells have been identified at all sites and comprise a majority of CD8$^+$ cells, the latter being particularly abundant in the transformation zone (117). A significant proportion of these cells express perforin and TIA-1 (119), suggestive of cytolytic capacity. In contrast, CD4$^+$ T cells are rarely found in these epithelia but predominate in the submucosa of the vagina, cervix and fallopian tubes. The submucosa of the vagina, cervix and fallopian tubes contains large numbers of J-chain- and IgA1- and IgA2-containing plasma cells, and the epithelium of the fallopian tubes and cervix stains for secretory component and has thus the potential to transport polymeric IgA (120, 121).

Vaginal immunization of human and non-human primates with cholera toxoid and CT, respectively, has been shown to evoke serum antibody responses and secretory IgA and IgG responses in cervico-vaginal washes (100, 122, 123). Further, in female macaques immunized with CT applied into the vagina, these responses were associated with large numbers of IgG and IgA antibody-secreting cells (ASCs) in the cervical and vaginal mucosae (100), demonstrating active production of such antibodies at local sites. However, this route of immunization was poorly if at all effective in inducing an IgA immune responses in other mucosal compartments (100).

Although all components of the mucosal immune system are present in the female reproductive tract, the precise sites of induction of secretory immune responses in this organ are largely unknown. The most likely scenario involves antigen uptake by intraepithelial Langerhans cells which then migrate to draining lymphoid tissues where they actively educate naive T and B cells. These in turn migrate via the efferent lymph and the blood circulation and seed to the submucosa where they differentiate into effector cells. Draining iliac lymph nodes appear to be the most likely site of initiation of such responses (92).

Thus, within the context of the common mucosal immune system, certain organs may favor the development of IgA-committed precursors B cells whose progeny is preferentially destined to particular mucosal locations. This new knowledge should call for more site-directed vaccination strategies.

Mucosal anti-infectious vaccines

Since Jenner introduced vaccination over 200 years ago, fewer than 50 vaccines have been approved for human use, nearly half of which are improved versions of earlier forms. All but five of the current vaccines – the oral poliovaccine, the oral killed whole cell/B subunit cholera vaccine, the oral *Salmonella typhi* vaccine, an oral rotavirus vaccine and nasal live-attenuated influenza virus vaccines which have completed phase III clinical testing – are administered parenterally and as such do not provide significant mucosal immunity.

It is now almost axiomatic that in order to be efficacious, vaccines against mucosal infections must stimulate the MALT, and that this goal is usually better achieved by administering immunogens by a mucosal route rather than parenterally. However, even so, stimulation of secretory immune responses by mucosal administration of most non-replicating antigens is often relatively inefficient, requiring multiple administrations of large quantities of immunogens and yielding most often tolerance and, if at all, modest immune responses. This conclusion is physiologically justifiable since the primary function of the mucosal immune system is to prevent the overstimulation of the entire immune system. It does so by eliminating mucosally applied antigens with denaturing acids, degradative enzymes and other innate factors as well as through intestinal peristalsis and ciliary movement on epithelia in the respiratory tract.

Against this background, mucosal vaccinologists have elaborated numerous delivery systems and adjuvants which partly circumvent such natural obstacles. The ever-growing list of mucosal delivery systems described in the literature bears witness to the difficulties encountered and to the limited practical usefulness of most delivery devices. We will therefore concentrate on the most promising systems. Furthermore, it should be emphasized that most of the results have been obtained in animal models rather than in humans with all unavoidable limitations.

Inert vaccine delivery systems

Because antigens are more immunogenic in particulate form than in solutions, and because they are vulnerable to antigen-degrading enzymes and acids, they have often been incorporated into vehicles that are by themselves non-toxic and non-immunogenic, and which protect vaccine material from degradation, enhance their uptake from mucosal surfaces, and may exhibit an adjuvant effect. Gelatin capsules coated with substances (e.g. cellulose acetate phthalate) that dissolve at alkaline pH in the intestine but not at acid pH in the stomach have been used for oral delivery of bacterial and viral vaccines in several studies performed in humans but their usefulness has never been systematically assessed (125–127).

To avoid the fast passage of free antigens, mucoadhesive polymers that adhere to mucosal surfaces and thus extend the time of exposure of vaccines have been used in several recent studies (123, 128, 129). When compounds such as highly viscous inert polysaccharide eldexomer (123) and carboxymethyl cellulose (128) are used with an antigen such as influenza virus or CT-B that has been orally, intranasally, or intravaginally administered, both local mucosal and systemic immune responses are induced. Other compounds that have been considered are carbapol, polycarboplil, sodium alginate, and hydroxypropyl cellulose, which are used in medicine for drug delivery.

Liposomes have been used for mucosal vaccination in a few studies, while bacterial or viral antigens have been administered orally or intranasally to rodents, monkeys and humans, resulting in the induction of mucosal and systemic immune responses (reviewed in (130)). A related antigen delivery system, cochleates, composed of protein–phospholipid–calcium precipitates with entrapped antigens, has been used for mucosal delivery of glycoproteins or peptides from influenza virus and SIV (131). As with liposomes, humoral as well as cell-mediated immunity (CMI) have been induced when given by the oral route.

Biodegradable microspheres composed of antigens incorporated into polymers of lactic and glycolic acid have been used far more extensively in mucosal vaccinology than any other inert delivery system (132–135). Microspheres have gained popularity because of their stability which allows them to protect incorporated antigens from acids and enzymes, and because of the ease with which their size and rate of biodegradation can be modified. Further, microspheres are by themselves non-antigenic and so can be easily reused, offering an advantage over live vectors. The rate of release of antigens can theoretically be controlled by mixing fast- and slow-releasing microspheres, allowing for the induction of combined primary and secondary immune responses by a single immunization (132). Finally, it would seem possible to incorporate immunoregulatory cytokines or DNA encoding for different antigens to achieve a desired immune response, and attempts to do so are in progress.

These obvious attractive features of the inert delivery systems are counterbalanced by serious disadvantages. Specifically, the disappointingly low absorption rate, usually much less than 1% of the ingested dose, renders the procedure expensive. Moreover, the use of organic solvents during the incorporation of antigens may severely compromise the immunogenicity of such vaccines. However, attempts are being made to increase the absorption rate and to develop conditions of incorporation that minimize degradation of antigens.

Mucosal lectin-like molecules as carrier-delivery systems

Oral administration of small amounts of protein antigens covalently coupled to carrier molecules with known affinity for mucosal epithelial cells, such as CT, *Escherichia coli* heat-labile toxin (LT), CT-B and LT-B, can elicit mucosal and also, under certain conditions, systemic antibody responses to the conju-

gated antigen (136–138). This strategy has also recently been utilized to induce mucosal immune responses to carbohydrate antigens (139).

Fusion proteins created from hybrids of CT-B or LT-B with genes encoding heterologous antigens have also been generated (140, 141), and are preferable to chemically coupled proteins in that they are free of contaminating holotoxin and are uniform in structure. Overexpression systems have also been developed to facilitate large-scale production of such fusion proteins (142). Others have created fusions between antigens and CT-B-CT-A2 mutants which lack the toxic A1 subunit (143–145). Interestingly, though oral administration induced serum IgG antibodies, maximum mucosal IgA responses required addition of the whole CT. Thus, it appears that CT-B is an excellent carrier molecule due to its ability to bind to GM1 receptors on epithelial cells but at best exhibit low adjuvant properties.

Live recombinant microorganisms as mucosal delivery sytems

It has long been assumed that only live vaccines would efficiently stimulate a mucosal immune response since the majority of microbial pathogens colonize or enter through mucosal surfaces and hence have evolved strategies to circumvent natural physico-chemical barriers. Furthermore natural infection with a number of microbial pathogens induce strong immune responses in both mucosal and systemic compartments and these responses are often protective against reinfection. The use of live attenuated recombinant bacteria and viruses, which can be genetically engineered to synthesize unrelated antigens, has the obvious advantage that it is theoretically possible to package the same recombinant organism with genes encoding several unrelated antigens. Recombinant bacterial and viral vectors which contain genes from unrelated pathogens that encode important virulence factors have been explored in many experimental vaccine studies (146). The development of recombinant vectors has been facilitated by rapid progresses relating to complementary DNA cloning of large DNA inserts and the advancement of polymerase chain reaction-based techniques. The ability of a vector to colonize specific mucosal locales and so to generate an immune response at desired sites is an important factor in determining its suitability as vaccine vehicle. The most important factor to be considered is the ability of a given vector to induce certain types of immune responses. Experience so far indicates that in general, mucosal administration of recombinant bacteria or viruses may induce mucosal S-IgA responses, and in most instances these responses are associated with priming of Th1-type helper responses which may not always be desirable. Although many bacterial and viral vectors have been used in animal models (147), only recombinant *Salmonella*, adenoviruses and poxviruses have been used with limited success in humans.

Salmonella presented by the oral route replicates in Peyer's patches and disseminates via the mesenteric lymph nodes to systemic sites, such as the spleen. This characteristic pattern of migration allows *Salmonella* to induce a broad-based immune response which includes cell-mediated as well as serum and mucosal antibody responses. Attenuated avirulent *Salmonella* strains have therefore received particular attention as mucosal vaccine delivery vectors for recombinant proteins associated with virulence (148–153). Although a large number of genes from bacteria, viruses, and parasites have been expressed in attenuated *Salmonella*, a critical balance between attenuation, adequate expression and immunogenicity has often been difficult to achieve.

Only recently have studies begun to characterize both mucosal T- and B-cell responses to recombinant antigens expressed in *Salmonella*, particularly in terms of the balance between antigen-specific $CD4^+$ Th1 and Th2 cells and their influence on the types of ensuing immune responses. Mice given an oral attenuated *Salmonella* vaccine displayed $CD4^+$ Th cells which produced IFN-γ and IL-2, but not IL-4 (154), immediately raising the question of as to how such T-cell help is also provided for mucosal S-IgA antibody responses. Oral delivery of rSalmonella which express the *Tox C* gene of tetanus toxoid (TT) resulted in predominant serum IgG2a and mucosal S-IgA antibody responses (62, 155). Splenic and Peyer's patch T cells selectively produced IFN-γ and IL-2 as well as the Th2-type cytokine IL-10. IL-4 was shown not to be involved in anti-*Salmonella* and anti-TT IgA responses by experiments in IL-4 knockout mice. Interestingly, $CD4^+$ Th cells in these IL-4 knockout mice exhibited two distinct cytokine patterns: a Th1-phenotype of IFN-γ and IL-2, as well as T cells which produced IL-6 and IL-10, but no IL-5 (155).

Intranasal immunization has emerged as perhaps the most effective route for induction of both peripheral and mucosal immunity to vaccines. The usefulness of *Mycobacterium bovis* strain Bacille Calmette Guérin (BCG) as a delivery system for recombinant antigens has been documented in several models. Specifically, BCG-vectored outer surface protein and pneumococcal protein A conferred protection against *Borrelia* and *S. pneumoniae*, respectively, when given nasally to mice (reviewed in (156)). Recently, attenuated *Bordetella pertussis* strains have been constructed by deleting the pertussis toxin gene and have been used as live vectors for intranasal delivery of heterologous antigens expressed at the surface of *B. pertussis* via fusion to the filamentous hemagglutinin (*FHA*) gene (157). Intranasal delivery

of B. pertussis expressing a parasite antigen fused to FHA induced strong mucosal immune response but relatively modest serum antibody responses to the fused antigen. Nevertheless, such an approach could be used for the construction of combined vaccines to protect not only against whooping cough but also against other respiratory infections.

Adenoviruses (r-Ad) have proven to be rather attractive systems for tissue targeted delivery of mammalian and microbial genes in gene therapy and in vaccinology, respectively. However, in both instances host immune responses develop to r-Ad and precludes readministration of the vector (158, 159). Despite these drawbacks, the use of r-Ad remains a promising approach in both arenas. Most studies to date have been performed with r-Ad from which the E1 and E3 genes have been deleted, rendering virus replication deficient. A major advantage of the current generation of r-Ad vectors is their capacity to accommodate large amounts of exogenous cDNA (7–8 kilobases), a feature which has been used to express virulence genes from a number of viral pathogens. However, the virions retain full competence for infection of mucosal epithelial cells in the respiratory tract (159, 160). In recent studies, r-Ad expressing herpes simplex virus glycoprotein B was shown to be a most effective vaccine when given by the intranasal route to mice (104, 105, 161). This regimen induced serum IgG as well as pulmonary IgA anti-glycoprotein B responses which correlated with protection from challenge. Further, this vaccine induced an effective CTL response (162). The same authors have also demonstrated that this vector induced specific secretory immune responses in the rodent female genital tract (104). This mucosal route of immunization also appears to be safe and effective, since intranasal, intratracheal and oral delivery of SIVenv glycoprotein by an r-Ad to macaques (163) and intranasal immunization of chimpanzees with a r-Ad expressing HIV *envelope* and *gag* genes (102) resulted in significant serum antibody responses. These antibody responses were broad-based, and when boosted with a gp160 subunit vaccine, elicited serum neutralizing antibodies. Recombinant Ad have also been used as vector for rabies virus in order to orally vaccinate foxes (164). In addition, oral administration of r-Ad expressing RSV and hepatitis B virus sequences have successfully induced circulating antibody responses in dogs and chimpanzees (165, 166). Moreover, intranasal or enteric immunization with a r-Ad expressing bovine coronavirus hemagglutinin-estrase glycoprotein induced virus specific mucosal immune responses in cotton rats (167). Despite these significant advances, further studies will obviously be required to determine optimal mucosal immunization protocols and to enhance the expression of desired antigens while diminishing responses to the vector to allow for vaccine readministration. In this respect, a recent study indicates that induction of mucosal tolerance (see below) to the r-Ad vector can be accomplished by prior mucosal exposure to inactivated adenoviral vector (168).

Recombinant poxviruses have been used in numerous studies including human clinical trials when given by systemic routes (by scarification, subcutaneously or intramuscularly), but studies documenting their mucosal immunogenicity are still limited. This is somewhat surprising given the effectiveness in wild animals of an oral vaccine against rabies (169) based on a recombinant vaccinia virus expressing the rabies glycoprotein gene (170). Oral administration of recombinant vaccinia virus encoding influenza virus hemagglutinin and neuraminidase induced both systemic and secretory anti-influenza antibody responses in mice (171, 172). Although relatively straightforward to generate in high yields and to purify, replication-competent recombinant vaccinia viruses pose potential problems for being used as mucosal vectors, including the risk of widespread infection in immunocompromised individuals. Vector systems based on avian poxviruses (fowlpox or canarypox-ALVAC) or an attenuated vaccinia virus (NYVAC), have now been developed which allow only single round infections in mammalian cells (173). Intranasal immunization of ferrets with NYVAC and ALVAC vectors expressing canine distemper virus *HA* and *F* genes were equally potent in protecting these animals against CDV challenge; intraduodenal administration was only partly effective (174). Parallel evaluation of different vector systems is rarely found in the litterature. In one study, the efficacy of r-Ad and NYVAC vectors expressing pseudorabies gD glycoprotein was compared after intranasal and intramuscular immunization. They have demonstrated that, while the adenovirus proved more efficient than the poxvirus vector to elicit an antibody response, the survival times of animals after challenge with pseudorabies virus was comparable (175). More recently, the modified vaccinia Ankara (MVA) strain of vaccinia virus has attracted the interest of many investigators. Indeed, MVA is a highly attenuated non-propagative strain of vaccinia which has been obtained after more than 500 consecutive passages in chicken embryo fibroblasts (176). The immunogenicity of recombinant MVA-based vectors by the parenteral route has recently been demonstrated (177) and MVA holds promises as a safe and effective mucosal vaccine delivery system. More generally, such non-replicative poxviral vectors are promising mucosal delivery systems.

The use of RNA viruses as vectors initially lagged behind the use of DNA viruses, mainly due to the relative ease of manipulating DNA genomes. Given the progress in recombinant DNA technology, the development of RNA viruses as vac-

cine vectors has now caught up with DNA viruses (reviewed in (178)). Recombinant vaccine strategies using RNA viruses such as poliovirus, alphaviruses (Venezuelan equine encephalitis virus (VEE), Semliki Forest virus), and paramyxoviruses (RSV) as vectors are being actively explored for mucosal immunization. In particular, replication-competent VEE expressing HIV p18 and p24 gag (179) or influenza virus *HA* genes (180) have been shown to induce mucosal and cellular immune responses. Interestingly, a replicon vaccine vector system was developed from an attenuated strain of VEE (181). The replicon RNA consists of the cis-acting 5' and 3' ends of the VEE genome, the complete non-structural protein gene region, and the subgenomic 26S promoter. Immunization of mice with the VEE replicon expressing the influenza *HA* or Lassa virus N gene induced antibody responses against the expressed protein and demonstrated a capability for sequential immunization to multiple pathogens in the same host (181). Likewise, recombinant poliovirus can be engineered as replication-competent vector (182) or as replicon (183) (reviewed in (178)). The unique aspects of these viruses, including their port of entry, point to promising developments in mucosal vaccinology.

Mucosal DNA vaccines

Since Wolff and colleagues (184) reported that direct intramuscular injection of mice with plasmid DNA encoding the complete sequence of a gene resulted in durable expression of the encoded protein, immunization with plasmid DNA encoding virulence genes of several important pathogens has been shown to induce protective immunity in several preclinical studies (185). Although many issues, including safety considerations, will need to be carefully assessed, this approach offers substantial advantages. Aside from stability and ease of production, these include the induction of immunity by a non-infectious agent, and thus the possibility of vaccinating against organisms with a propensity for long-term persistence in the body, e.g. cytomegalovirus and HIV. Furthermore, DNA immunization would allow the selection of nucleic acids corresponding to a broad range of polypeptides to overcome the problem of MHC genetic restriction in outbred populations. In addition, the plasmid backbone can also be equipped to harbour sequences encoding cytokine genes so as to facilitate the type of immune response desired.

In principle, the DNA used for vaccination is a plasmid containing a bacterial origin of replication and an antibiotic resistance gene, required for selective plasmid production in *E. coli*. The gene of interest is cloned under the control of a strong promoter (usually a viral promoter). In addition to the expression cassette, the plasmid may contain immunostimulatory sequences which appear to be critical in governing the immunogenicity of the expressed gene. These sequences include a non-methylated CpG motif within a palindromic hexamer oligonucleotide sequence (186, 187) and have been reported to induce B-cell proliferation (188) and Th1 cytokine production (189, 190). The stimulatory properties of such motifs appears to be mediated via the selective activation of DCs (191). It is interesting to note that mucosal administration of synthetic oligodeoxynucleotides containing CpG motifs enhances virus specific responses, suggesting that such nucleotides could be used also as mucosal adjuvant (192).

Although the majority of studies so far have dealt with intracutaneous and intramuscular administration of DNA vaccines, this approach has gained popularity among mucosal vaccinologists. Naked plasmid DNA can be directly used for mucosal immunization (193–197). Thus, intranasal or intratracheal administration of a DNA plasmid encoding influenza virus hemagglutinin induced protection against a lethal influenza virus challenge in mice (185). More recently, intranasal immunization of mice with a DNA plasmid encoding lacZ and herpes simplex virus type 1 glycoprotein B (gB) induced lacZ and gB expression in lungs and cervical lymph nodes (195). Although a distal mucosal IgA response and an anti-HSV cell-mediated immune response were observed following three i.n. administrations of gB DNA, protection against lethal vaginal HSV challenge was lower than that seen after intramuscular administration of the same plasmid. Intrabuccal injection or nasal administration of plasmid DNA encoding measles virus hemagglutinin induced systemic MHC class I-restricted CTL responses (196). Further, intravaginal instillation of a plasmid DNA encoding the HIV-1 envelope glycoprotein elicits production of IgA and IgG antibodies in vaginal fluids and serum neutralizing antibodies (194). The same construct was employed to demonstrate that plasmid DNA vaccination of infant chimpanzees was well tolerated and could induce serum antibodies to HIV-1 (197).

As for conventional vaccines, several formulations have been developed to facilitate the uptake of plasmid DNA-based vaccines and to protect them from degradation in the mucosal microenvironment. The use of liposomes, cationic lipids, monophosphoryl lipid A for mucosal delivery of DNA (198–201) has been shown to increase both expression and immunogenicity of the corresponding protein, suggesting that the lipid matrix may also provide a secondary role as adjuvant by facilitating DNA uptake by APCs or by promoting local inflammation. In the case of oral delivery, plasmid DNA can also be protected from degradation in the gut by encapsulation

in poly(DL-lactide-co-glycolide) (PGL) microparticles (202, 203).

Attenuated intracellular bacteria such as *Salmonella*, which are retained within vacuoles in the infected cell, have recently shown promise for the delivery of plasmid DNA. Thus, infection of peritoneal macrophages with *S. typhi* carrying a plasmid encoding lac Z results in the transfer of plasmid DNA into the infected cell (204). Very recently, this approach has been employed to demonstrate that oral administration of *S. typhi* carrying a tumor antigen results in gene transfer in host DCs and protects mice against fibrosarcoma (205).

The mechanisms underlying the induction of an immune response after mucosal DNA vaccination are still unknown, but are likely to be analogous to those involved after systemic (intramuscular, intradermal) DNA administration (206–209): transfer of antigen from tissue-transfected epithelial cells to professional mucosal APCs (cross-priming), or direct transfection of professional APCs and especially DCs.

Edible vaccines

Mucosal vaccines would be more widely used – especially in developing countries – if they could be produced at lower cost, and distributed without refrigeration. The concept of vaccine production in transgenic plants was introduced recently (210), and was based on the premise that plants have the capacity to produce abundant biomass, and that recombinant immunogens could be produced in plant tissues. Since plants can be engineered to contain multiple foreign genes, multicomponent transgenic plant vaccines should therefore be feasible. Several plant expression systems, e.g. potato and tobacco plants, have been developed and shown to allow expression of microbial antigens such as hepatitis B surface antigen, E. coli LT-B, and even Norwalk Virus-like particles (VLPs) (reviewed in (211)). Although the levels of expression of recombinant antigens have so far been very disappointing, feeding animals with potato tubers expressing Norwalk VLPs or E. coli LT-B induced specific secretory IgA antibody responses and serum IgG responses to the transgenic protein (211). Developing strategies to increase antigen expression in edible plants, including the use of strong tissue-specific promoters remain one important challenge in this area of vaccine development.

Dendritic cells as vaccines against mucosal infections

DCs are potent APCs that play a central role in the induction of immunity as initiators and immunomodulators of immune responses (212). Owing to their capacity to prime T cells, DCs loaded *ex vivo* with tumor antigens have been extensively used as potential cancer vaccines (213, 214). Given promising preclinical results, studies are now underway in patients. That adoptive transfer of DCs pulsed *ex vivo* with infectious microorganisms could constitute a way to induce broad-based immunity against infectious diseases has also been documented (215–217).

To date, the potential of DCs as vaccine carriers for the induction of mucosal responses is largely unknown. In this respect, a recent study has shown that DCs pulsed *ex vivo* with killed chlamydiae and subsequently transferred into naive recipients were able to induce protective $CD4^+$ Th1 immune responses against genital challenge with chlamydia (218). Although this study used bone marrow-derived DCs, it is the first one to document the efficacy of this approach against a mucosal infection.

Bacterial enterotoxins as mucosal adjuvants

The introduction of proteins including vaccines into mucosal inductive sites is an effective way to induce systemic unresponsiveness (mucosal tolerance). Thus, mucosal adjuvants are required not only to boost mucosal and systemic immunity, but also to prevent the induction of mucosal tolerance.

Although a variety of compounds have been reported to display adjuvant properties on mucosally co-administered antigens, the most powerful and hence most studied mucosal adjuvants are the *Vibrio choleme* exotoxin CT, and its structural and biological analog LT. These macromolecules are composed of two structurally, functionally and immunologically separate A and B subunits (219). The B subunit of both toxins consists of five identical monomers, but the pentameric B subunit of CT (CT-B) binds only to GM1 ganglioside, while the B subunit pentamer of LT (LT-B) is more promiscuous and binds to GM1 as well as to asialo GM1, GM2, and glycoprotein receptors (220). After the B subunit binds to epithelial cell GM1 or GM2 receptors, the A subunit reaches the cytosol and binds to NAD to catalyze ADP-ribosylation of $G_{s\alpha}$. This GTP-binding protein activates adenyl cyclase with subsequent elevation of cAMP, which in epithelial cells results in secretion of water and chloride ions into the small intestine.

Mucosal exposure to CT and LT, which are both immunogenic, results in S-IgA and serum IgG antibodies, which are almost entirely restricted to CT-B or LT-B. More importantly, both toxins are potent mucosal adjuvants for unrelated proteins co-administered by oral, intranasal or even parenteral routes (221–223).

Recent studies suggest that oral or nasal immunization with proteins along with CT as adjuvant induces Th2-type responses in the intestinal mucosa (224–226). Such an immu-

nization protocol induced CD4+ Th2 cells in Peyer's patches and the spleen (224) as well as high IgG1 and IgE responses, in the absence of detectable IgG2a titers (226, 227). Further, oral immunization with keyhole limpet hemocyanin (KLH) and CT mixed with CT-B resulted in Peyer's patch and lamina propria T-lymphocyte populations which produced low IL-2 and IFN-γ but high levels of IL-4 and IL-5 (228).

However, other studies have shown that oral co-administration of a soluble or particulate antigen and CT primed animals for systemic Th1-type immune responses (delayed-type hypersensitivity) (229) and induced functional CTLs in the spleen (230). Again, the results from these studies suggest that oral immunization with heterologous antigen and CT as an adjuvant can differentially affect mucosal and systemic immune responses, inducing Th2-type immune responses in mucosal tissues and Th2 as well as Th1-type responses in the systemic compartment. Thus, not only the nature of the adjuvant, but also that of the antigen used, and the mucosal route of delivery can all influence whether Th1- and/or Th2-type responses develop.

CT and LT cause severe diarrhea in humans and account for the clinical manifestations of cholera and enterotoxigenic *E. coli* enteritis, and thus neither is suitable for use as an enteric adjuvant in humans. Early studies attempted but failed to dissociate diarrhoeagenicity from the adjuvanticity of these two molecules. For example, a non-toxic mutant, formed by making a single amino acid substitution in the ADP ribosyltransferase active center also lacked adjuvanticity when administered orally (231). However, mutants of LT formed by substituting a single amino acid either in or outside the ADP-ribosyltransferase cleft, retained adjuvanticity despite apparent lack of toxicity when administered by the intranasal route (108, 232–234). Moreover, two mutants of CT, which harbor single amino acid substitutions in the ADP-ribosyltranferase active center and completely lack ADP-ribosyltransferase activity and diarrheagenicity, remained effective adjuvants and were comparable to native CT when given parenterally or nasally (235, 236). We have ourselves found that recombinant CT-B and LT-B are also moderately adjuvant when co-administered with other protein antigens by the nasal route but not by the oral route (C. Czerkinsky, J. Holmgren, unpublished observations). Since enterotoxicity is the main obstacle to the use of native CT or LT as oral adjuvants, it will be interesting to determine whether enzymatically inactive CT and LT mutants have retained adjuvant activity when given orally.

Mucosal tolerance and anti-inflammatory vaccines

One of the primary goals in developing effective therapies against diseases caused by unwanted or tissue damaging inflammatory immune responses, is to specifically suppress or decrease to an acceptable level the intensity of untoward immune reactions without affecting the remainder of the immune system. Induction of tolerance in mature pathogenic T cells represents an ideal form of specific immunotherapy in the treatment of chronic inflammatory autoimmune disorders and allergies.

Three main modes of peripheral tolerance induction by antigens have been considered: parenteral administration of antigens, parenteral administration of antigen analogs which act as T-cell receptor antagonists, and mucosal administration of antigens or so-called "oral tolerance". Mucosal administration of antigen is in fact a long-recognized method of inducing peripheral tolerance. The phenomenon, often referred to as "oral tolerance" (because it was initially documented by the effect of oral administration of antigen), is characterized by the fact that animals fed or having inhaled an antigen become refractory or have diminished capability to develop an immune response when re-exposed to the same antigen introduced by the systemic route, e.g. by injection. This effect is especially pronounced for Th1 cell-mediated immune responses and is regarded as an important natural physiological mechanism whereby we avoid developing delayed-type hypersensitivity reactions to dietary and airborne antigens, and products from commensal microorganisms. Mucosally induced immunological tolerance can affect all types of adaptive immune responses, depending on the animal species, the age, the form and dose of antigen, and the route of mucosal administration (enteric, buccal, nasal, rectal, genital).

Mechanisms of tolerance after mucosal delivery of antigens

Mucosal uptake of antigens may result in the development of immunity or tolerance, or even both, the decision being taken in the epithelium or underlying lymphoid tissue and being mainly determined by the nature and physico-chemical form of the antigen.

Depending upon the dose of antigen administered, deletion or anergy of antigen-specific T cells and/or expansion of cells producing immunosuppressive cytokines (IL-4, IL-10 and TGF-β) (reviewed in (73)), may result in decreased T-cell responsiveness. It is interesting to note that the latter scenario involves cytokines that are also known to promote IgA isotype switching and IgA production, and is thus compatible with the observation that secretory IgA antibody responses and systemic

T-cell tolerance may develop concomitantly (237). Because tolerance can be transferred by both serum and cells from tolerized animals, it is possible that humoral antibodies (IgA?), circulating undegraded antigens or tolerogenic fragments and cytokines may act synergistically to confer T-cell unresponsiveness. Without excluding the above possibilities, another mechanism that may be considered could involve antigen-driven attraction of inflammatory T cells from the periphery into the mucosal microenvironment where they could be rendered anergic, functionally skewed, deleted or even ignored. This form of antigen-driven "anatomic deviation" would imply that inflammatory T cells activated in peripheral tissues exhibit a promiscuous migratory behaviour allowing their entry not only in systemic organs but also in mucosal tissues at the site of antigen uptake. The latter scenario has not yet been addressed experimentally but is compatible with studies showing that arthritogenic and diabetogenic T cells do express the cell surface mucosal integrin $\alpha4\beta7$ whose ligand, the mucosal addressin cellular adhesion molecule (MadCAM-1), is also expressed on inflamed pancreas and synovial tissues. In this respect, it is also interesting to note that oral tolerization with a prototype soluble protein antigen has recently been shown to differentially affect the production by peripheral T cells of the β (C-C) chemokines MIP-1α and MCP-1 (238), which are potent chemoattractants for T cells.

Irrespective of effector mechanism(s) involved, a major question that arises is where and how tolerance is induced, be it suppression, anergy, deletion, ignorance and/or anatomical deviation. To date, very little is known regarding the mechanisms governing induction of mucosal tolerance, and especially the intracellular pathways of entry of tolerogens, the nature of APC elements involved, their tissue localization, and the characteristics of signals transduced from such cells to responding T cells.

At variance with systemically administered antigens, antigens handled in mucosal tissues have already been subject to a variety of innate factors such as, e.g., proteases, acids, salts, *mucins*, that have altered their form prior to uptake. As a result of this "extratissular conditioning" different epitopes may be exposed and their uptake and/or processing may involve many different cell types.

The observation that mucosally induced systemic tolerance depends on an intact epithelial barrier (239, 240) suggests a central role for the epithelium. M cells have been shown to uptake a variety of particulate antigens such as viruses and bacteria, and to allow direct entry of invasive microorganisms in mucosal inductive sites. Such a pathway is thought to result in the induction of secretory IgA immune responses. Although the ability of M cells to serve as APCs appears to be poorly supported, these cells could still theoretically be involved in an abortive form of antigen presentation leading to tolerance induction. The role of absorptive epithelial cells, such as intestinal enterocytes, in tolerance induction has been underscored by several studies (reviewed in (241, 242)). Epithelial enterocytes express co-stimulatory molecules such as non-classical MHC class I (CD1d) molecules involved in antigen presentation to subpopulations of T cells and abnormal forms or levels of MHC class II molecules, leading to selective triggering of suppressive $CD8^+$ T cells and/or abortive presentation to $CD4^+$ T cells. In addition, epithelial enterocytes have also been shown to produce cytokines, such as IL-10 and TGF-β, which are particularly efficient at suppressing the inductive phase of $CD4^+$ T-cell-mediated responses.

All known types of classical APCs, including DCs, macrophages and B cells, and even mast cells, have been shown to populate mucosal tissues but, because of their heterogeneity and of the difficulty in isolating pure subpopulations of APCs from mucosal tissues, their respective role in inducing tolerance has not yet been elucidated. Although activated B cells and tissue macrophages are powerful APCs for memory Th cells, evidence suggests that antigen presentation by resting B cells results in T-cell tolerance (243, 244). It should however be noted that B cells activated *in vitro* with bacterial lipopolysaccharide (LPS), a prominent component of the normal mucosal microflora, are capable of inducing tolerance when injected into naive hosts (244). Although functional DCs have been identified in mucosal tissues such as the Peyer's patches, the mesenteric lymph, the intestinal lamina propria and the airway mucosa (reviewed in (245)), their role in activating rather than suppressing naive T cells has received strongest support. Thus, DCs freshly isolated from the airway mucosa preferentially activate Th2 cells but can also mature into APCs capable of activating Th1 (246). Thus, airway DC cells could play a pivotal role in the cross-regulation of Th1- and Th2-driven responses. However, recent experiments with splenic dendritic cells have shown a differential capacity of these DC subpopulations to activate T cells, suggesting the existence of regulatory and immunostimulatory DC subsets, supporting the emergence of the concept of tolerogenic DCs (247). Further, treatment with Flt3 ligand which is known to expand DCs *in vivo*, has been reported to enhance oral tolerance (248). Interestingly, LPS, which is known to cause the rapid exit of DCs, has also been shown to enhance tolerance induction (249). This information places strong emphasis on the site of entry and on the intracellular pathway of processing of antigens administered to a mucous membrane in the induction of tolerance and/or immunity and calls for the need to develop vaccine formula-

tions with intrinsic immunomodulating and cellular targetting properties.

Mucosal immunotherapy: potential and limitations

Since mucosally induced immunological tolerance is exquisitely specific to the antigen initially ingested or inhaled, and thus does not influence the development of systemic immune responses against other antigens, its manipulation has become an increasingly attractive strategy for preventing and possibly treating illnesses associated or resulting from the development of adverse immunological reactions against self and non-self antigens. The approach has been considered for preventing or treating hypersensitivities to common allergens (250–252).

The phenomenon of mucosally induced systemic tolerance has likewise been utilized to suppress immune responses against self antigens. It has thus been possible to delay the onset and to decrease the intensity of experimental autoimmune diseases in a variety of animal systems by mucosal deposition of auto-antigens onto the intestinal (by feeding) or the respiratory mucosa (by aerosolization or intranasal instillation of antigens) (reviewed in (73)). For instance, oral administration of collagen type II has been shown to delay the onset of autoimmune arthritis. Similarly, it has been possible to suppress an experimental form of autoimmune uveoretinitis by oral administration of the retinal S-antigen.

Although the above examples indicate that mucosal administration of foreign as well as self antigens offers good promise for inducing specific immunologic tolerance, the applicability of this approach in human medicine remains limited by practical problems. Indeed, to be clinically broadly applicable, mucosally induced immunological tolerance must also be effective in patients in whom the disease process has already established itself and/or in whom potentially tissue-damaging immune cells already exist. This is especially important when considering strategies of tolerance induction in patients suffering from or prone to an autoimmune disease or an allergic condition. Current protocols of mucosally-induced tolerance have had limited success in suppressing the expression of an already established state of systemic immunological sensitization (253, 254). This may partly explain the disappointing results of recent clinical trials of oral tolerance in patients with multiple sclerosis and rheumatoid arthritis.

In addition, and by analogy with mucosal anti-infectious vaccines, induction of mucosal tolerance requires administration of massive amounts of antigens or prolonged administration of relatively smaller amounts of antigens which are then only effective in rather narrow dose ranges. As for conventional vaccines, efforts have recently been devoted to the construction of novel or improved formulations to induce mucosal tolerance. In this respect, the use of mucosal lectins endowed strong immunomodulating properties, such as cholera and E. coli heat-labile enterotoxin B subunits, as carrier for mucosal delivery of tolerogenic peptides has received considerable attention during the past five years as described below.

Cholera toxin B subunit as mucosal carrier-immunomodulating system for antipathological vaccination

Recent studies have shown that physical coupling of an antigen to CT-B led to unexpected effects: when given by various mucosal routes, CT-B induced a strong mucosal IgA immune response to itself and in some cases also to the conjugated antigen, but instead of abrogating systemic tolerance enhanced it profoundly (229). Based on this finding and on the results of other experiments with a variety of antigens (255), there is good reason to believe that such a system may be advantageous for inducing peripheral tolerance. First, it minimizes by several hundred-fold the amount of antigen/tolerogen and drastically reduces the number of doses that would otherwise be required by reported protocols of orally-induced tolerization. Second, but most important, this strategy appears to be applicable for suppressing the expression of an already established state of systemic immune sensitization. Here, we shall summarize the results of studies using this approach as a means to prevent or treat pathological immune responses associated with experimental autoimmune diseases, type I allergies, and allograft rejection.

Treatment of organ-specific autoimmune diseases

Mucosal administration of relevant autoantigens linked to CT-B could inhibit the development of clinical disease in animal models of experimentally inducible autoimmune diseases, such as allergic encephalomyelitis (256) and collagen-induced arthritis (257). In the latter model, nasal administration of a collagen type II–CT-B conjugate could inhibit disease progression, even when treatment was initiated after onset of clinically overt disease. Furthermore, oral treatment of female NOD mice with a CT-B–insulin conjugate could suppress type I diabetes (258), a model of spontaneous autoimmune disease, even when given as late as 15 weeks post-birth (that is at a time when all mice have evidence of insulitis). Taken together, these observations indicate that CT-B-driven mucosal tolerance can affect not only the afferent but also the efferent phase of systemic T-cell-mediated inflammatory responses.

Depending on the nature of the conjugated antigen, the route of administration (oral, nasal) of the conjugate, and the animal species used, this type of treatment variably affected the capacity of lymph node T cells to produce Th1 or Th2 cytokines.

The most striking observation in all three models of autoimmune diseases tested was the finding that treatment with CT-B-antigen suppressed leukocyte infiltration into the target organ. This suggests that the mechanisms governing induction of tolerance by feeding or inhaling CT-B-linked antigens may involve modifications of the migratory behavior of inflammatory cells.

Prevention of graft rejection

By coupling thymocytes to cholera B subunit and feeding this conjugate to mice, it has been possible to significantly prolong the survival of transplanted hearts in allogeneic mouse recipients (unpublished results). Recently, feeding CT-B-derivatized donor keratinocytes has been shown to prevent corneal allograft rejection in mice (259).

Prevention of type I allergies

The possibility of preventing type I allergic reactions by mucosal administration of a prototype allergen linked to a mucosal vector with intrinsic immunomodulating properties has also recently been examined in a mouse model of ovalbumin (OVA)-induced allergic reactions. Thus, mice nasally administered with OVA conjugated to *E. coli* LT-B prior to allergic sensitization showed suppressed skin DTH responses to OVA and also suppressed serum IgE antibody responses to the inhaled allergen (260). Further, these mice showed markedly decreased anaphylactic responses to intravenously administered OVA. Taken together, the latter observations indicate that under certain conditions, mucosal administration of soluble protein together with an immunomodulating mucosal vector can suppress both systemic Th1- and Th2-driven responses. The fact that the same type of regimen is also known to favor S-IgA responses in mucosal tissues makes this concept even more attractive since IgA is known to be non-phlogistic and could theoretically outcompete IgE for binding to a given allergen. However, it should also be pointed out that suppression of Th2-driven responses such as IgE-antibody responses appears considerably more difficult to achieve than corresponding Th1 responses (e.g. DTH) in an animal already systemically sensitized to the allergen; in the latter situation, mucosal treatment with CT-B-conjugated allergen required prolonged administration of the conjugate and was effective only with certain allergens (C. Rask, J. Holmgren, C. Czerkinsky, unpublished observations).

Mucosal vaccines for simultaneous induction of anti-infectious and antipathological immunity

Somewhat surprisingly, vaccinologists in general and mucosal immunologists in particular have usually believed that a reciprocal relationship exists between induction of immunity and tolerance. The observation that mucosal immunity, which is typified by secretory IgA antibodies, may develop concomitantly with systemic immunological tolerance has led to the belief that vaccines against mucosal pathogens should primarily stimulate immunity without inducing tolerance. However, from a theoretical standpoint, the possibility to manipulate the mucosal immune system towards both immunity and tolerance appears rather attractive when considering strategies aimed at protecting the host from colonization or invasion by mucosal pathogens but also to interfere with the development of potentially harmful systemic immunological reactions against the same pathogens or their products.

The notion that immunological tolerance may provide the host with a protective mechanism against an infectious disease has been elegantly illustrated by recent studies in transgenic mice. Whereas mice from a susceptible (BALB/c) background develop an early Th2-driven IL-4 response and ultimately succumb to their infection with *Leishmania major*, mice rendered tolerant by transgenic expression in the thymus of LACK, a protective surface antigen of *Leishmania*, fail to produce this early response and resolve their infection (261). Very recently, tolerization of post-thymic, mature parasite-specific T cells could also be accomplished in the periphery after nasal administration of as little as 10 μg of *L. major* LACK antigen conjugated to CT-B (262). Such treatment markedly delayed the onset of lesion development in infected mice and reduced parasite burden in the skin and draining lymph nodes of infected mice.

Similar findings have also been observed in mice that had already been infested with the parasitic trematode, *Schistosoma mansoni* (263) and treated with a CT-B–parasite conjugate vaccine. Thus, nasal treatment of mice with *S. mansoni* glutathione S-transferase (GST) conjugated to CT-B suppressed granuloma formation and decreased parasite burden and egg deposition in the liver of infested animals. Protection with this nasal CT-B–GST vaccine was associated with decreased hepatic production of IFN-γ, IL-5 and IL-3 but apparently intact IL-4 production. Most importantly, such treatment could significantly prolong the survival of animals, even when initiated as late as 6 weeks after initial infection, that is at a time when liver granulomatous reactions are most pronounced.

While this type of approach has only been attempted in two parasitic diseases, there are obvious microbial diseases which could theoretically benefit from the concomitant induction of S-IgA immune responses and downregulation of local T-cell-driven immunopathology. Examples of such diseases include gastroduodenal ulcers caused by *Helicobacter pylori*, genital ulcers caused by papilloma viruses, broncho-pneumonitis

Table 1. **Mucosal vaccines**

Anti-infectious vaccines	Anti-inflammatory vaccines
Enteric infections	Chronic inflammation caused by microorganisms
Genitourinary infections	Autoimmune diseases
Respiratory infections	Allergies
Occular, buccal and ear infections	Allograft rejection

induced by parainfluenzae viruses and respiratory syncytial virus, or chronic pelvic inflammaory disease, trachoma and urethritis caused by *Chlamydia pneumoniae*.

Other mucosal delivery systems for induction of tolerance

Most of the work published so far on induction of peripheral tolerance after mucosal administration of antigens has involved the use of free antigens and more recently that of antigens co-administered with CT-B or analogs. However, the possibility of developing improved tolerogenic formulations based on expression of selected antigens and/or immunomodulating cytokines and chemokines in appropriate vectors, e.g. lactobacilli, edible plants (264), or corresponding genes in plasmid DNA (265), are now being actively addressed.

Mucosally induced tolerance has the virtue of being a powerful natural and specific protective mechanism against adverse immune reactions that may result from mucosal intake of immunogenic matters. This property may be utilized to treat disorders associated with untoward immune responses to self and non-self antigens, such as certain autoimmune diseases, allergic reactions, and graft rejection (*Table 1*). Hence, mucosal immunomodulation via appropriate delivery of tolerogenic compounds may be where the future of anti-inflammatory vaccines lies.

References

1. Liew FY, Russell SM, Appleyard G, Brand CM, Beale J. Cross-protection in mice infected with influenza A virus by the respiratory route is correlated with local IgA antibody rather than serum antibody or cytotoxic T-cell reactivity.
Eur J Immunol 1984;**14**:350–356.
2. Mazanec MB, Kaetzel CS, Lamm ME, Fletcher D, Nedrud JG. Intracellular neutralization of virus by immunoglobulin A antibodies.
Proc Natl Acad Sci USA 1992;**89**:6901–6905.
3. Alley CD, Mestecky J. The mucosal immune system. In: Birdand G, Calvert JE, eds. B lymphocytes in human disease. Oxford: Oxford University Press; 1988. p. 222–254.
4. Allansmith MR, McClellan BH, Butterworth M, Maloney JR. The development of immunoglobulin levels in man.
J Pediatr 1968;**72**:276–290.
5. Mellander L, Carlsson B, Hanson LA. Appearance of secretory IgM and IgA antibodies to *Escherichia coli* in saliva during early infancy and childhood.
J Pediatr 1984;**104**:564–568.
6. Brandtzaeg P. The role of J chain and secretory component in receptor-mediated glandular and hepatic transport of immunoglobulins in man.
Scand J Immunol 1989;**22**:111–146.
7. Mestecky J, Lue C, Russell MW. Selective transport of IgA: cellular and molecular aspects.
Gastroenterol Clin North Amer 1991;**20**:441–471.
8. Guy-Grand D, Griscelli C, Vassalli P. The mouse gut T lymphocyte, a novel type of T cell. Nature, origin, and traffic in mice in normal and graft-versus-host conditions.
J Exp Med 1978;**148**:1661–1667.
9. Davies MD, Parrott DM. The early appearance of specific cytotoxic T cells in murine gut mucosa.
Clin Exp Immunol 1980;**42**:273–279
10. MacDermott RP, Franklin GO, Jenkins KM, Kodner IJ, Nash GS, Weinrieb IJ. Human intestinal mononuclear cells. I. Investigation of antibody-dependent, lectin-induced and spontonaeous cell-mediated cytotoxic capabilities.
Gastroenterology 1980;**78**:47–56.
11. Davies MD, Parrott DM. Cytotoxic T cells in small intestine, epithelial, lamina propria and lung lymphocytes.
Immunology 1981;**44**:367–371.
12. Tagliabue A, Luini W, Soldateschi D, Boraschi D. Natural killer activity of gut mucosal lymphoid cells in mice.
Eur J Immunol 1981;**11**:919–922.
13. Nauss KM, Pavlina TM, Kumar V, Newberne PM. Functional characteristics of lymphocytes isolated from the rat large intestine. Response to T-cell mitogen and natural killer cell activity.
Gastroenterology 1984;**86**:468–475.
14. Ernst PB, Befus AD, Bienenstock J. Leukocytes in the intestinal epithelium: An unusual immunologic compartment.
Immunol Today 1985;**6**:50–55.
15. Smyth MJ, Trapani JA. The relative role of lymphocyte granule exocytosis versus death-receptor-mediated cytotoxicity in viral pathophysiology.
J Virol 1998;**72**:1–9.
16. London SD, Rubin DH, Cebra JJ. Gut mucosal immunization with reovirus serotype 1/L stimulates virus-specific cytotoxic T cell precursors as well as IgA memory cells in Peyer's patches.
J Exp Med 1987;**165**:830–847.
17. Offit PA, Cunningham SL, Dudzik KI. Memory and distribution of virus-specific cytotoxic T lymphocytes (CTLs) and CTL precursors after rotavirus infection.
J Virol 1991;**65**:1318–1324.
18. London SD, Cebra-Thomas JA, Rubin DH, Cebra JJ. CD8 lymphocyte subpopulations in Peyer's patches induced by reovirus serotype 1 infection.
J Immunol 1990;**144**:3187–3194.
19. George A, Kost SI, Witzleben CL, Cebra JJ, Rubin DH. Reovirus-induced liver disease in severe combined immunodeficient (SCID) mice. A model for the study of viral infection, pathogenesis, and clearance.
J Exp Med 1990;**171**:929–934.
20. Cuff CF, Cebra CK, Rubin DH, Cebra JJ. Developmental relationship between cytotoxic $\alpha\beta$ T-cell-receptor-positive intraepithelial lymphocytes and Peyer's patch lymphocytes.
Eur J Immunol 1993;**23**:333–1339.
21. Issekutz TB. The response of gut-associated T lymphocytes to intestinal viral immunization.
J Immunol 1984;**133**:2955–2960.

22. Buzoni-Gatel, D, Lepage, AC, Dimier-Poisson, IH, Bout, DT, Kasper LH. Adoptive transfer of gut intraepithelial lymphocytes protects against murine infection with *Toxoplasma gondi*. J Immunol 1997;**158**:5883–5889
23. Offit PA, Dudzik KI. Rotavirus-specific cytotoxic T lymphocytes passively protect against gastroenteritis in suckling mice. J Virol 1990;**64**:6325–6328.
24. Dharakul T, et al. Immunization with baculovirus-expressed recombinant rotavirus proteins VP1, VP4, VP6 and VP7 induces CD8+ T lymphocytes that mediate clearance of chronic rotavirus infection in SCID mice. J Virol 1991;**65**:5928–5932.
25. Franco MA Greenberg HB. Role of B cells and cytotoxic T lymphocytes in clearance of and immunity to rotavirus infection in mice. J Virol 1995;**69**:7800–7806.
26. Franco MA, Tin C, Rott LS, VanCott JL, McGhee JR, Greenberg HB. Evidence for CD8+ T-cell immunity to murine rotavirus in the absence of perforin, fas and γ interferon. J Virol 1997;**71**:479–486.
27. Burns JW, Siadat-Pajouh M, Krishaney AA, Greenberg HB. Protective effect of rotavirus VP6-specific IgA monoclonal antibodies that lack neutralizing activity. Science 1996;**272**:104–107.
28. Belyakov IM, et al. Mucosal immunization with HIV-1 peptide vaccine induces mucosal and systemic cytotoxic T lymphocytes and protective immunity in mice against intrarectal recombinant HIV-vaccinia challenge. Proc Natl Acad Sci USA 1998;**95**:1709–1714.
29. Anderson MJ, Pattison JR, Cureton RJ, Argent S, Heath RB. The role of host responses in the recovery of mice from Sendai virus infection. J Gen Virol 1980;**46**:5052–5060.
30. Johnson RA, Prince GA, Suffin SC, Horswood RL, Chanock RM. Respiratory syncytial virus infection in cyclophosphamide-treated cotton rats. Infect Immun 1982;**37**:369–373.
31. Bender BS, Croghan T, Zhang L, Small PA Jr. Transgenic mice lacking class I major histocompatibility complex-restricted T cells have delayed viral clearance and increased mortality after influenza virus challenge. J Exp Med 1992;**175**:1143–1145.
32. Crowe JE Jr. Host responses to respiratory virus infection and immunization. Curr Top Microbiol Immunol 1999;**27**:191–214.
33. Allan W, Tabi Z, Cleary A, Doherty PC. Cellular events in the lymph node and lung of mice with influenza. Consequences of depleting CD4+ T cells. J Immunol 1990;**144**:3980–3986.
34. Eichelberger M, Allan W, Zijlstra M, Jaenisch R, Doherty PC. Clearance of influenza virus respiratory infection in mice lacking class I major histocompatibility complex-restricted CD8+ T cells. J Exp Med 1991;**174**:875–880.
35. Carding SR, Allan W, Kyes S, Hayday A, Bottomly K, Doherty PC. Late dominance of the inflammatory process in murine influenza by γδ+ T cells. J Exp Med 1990;**172**:1225–1231.
36. Muñoz JL, McCarthy CA, Clark ME, Hall CB. Respiratory syncytial virus infection in C57BL/6 mice: Clearance of virus from the lungs with virus-specific cytotoxic T cells. J Virol 1991;**65**:4494-4497.
37. Nicholas JA, Rubino KL, Levely ME, Meyer AL, Collins PL. Cytotoxic T cell activity against the 22-kDa protein of human respiratory syncytial virus (RSV) is associated with a significant reduction in pulmonary RSV replication. Virology 1991;**182**:664–672.
38. Graham BS, Bunton LA, Wright PF, Karzon DT. Role of T lymphocyte subsets in the pathogenesis of primary infection and rechallenge with respiratory syncytial virus in mice. J Clin Invest 1991;**88**:1026–1033.
39. Graham BS, Henderson GS, Tang YW, Lu X, Neuzil KM, Colley DG. Priming immunization determines T helper cytokine mRNA expression patterns in lungs of mice challenged with respiratory syncytial virus. J Immunol 1993;**151**:2032–2040.
40. Miller CJ. Mucosal transmission of simian immunodeficiency virus. Curr Top Microbiol Immunol 1994;**188**:107–122.
41. Lohman BL, Miller CJ, McChesney MB. Antiviral cytotoxic T lymphocytes in vaginal mucosa of simian immunodeficiency virus-infected rhesus macaques. J Immunol 1995;**155**:5855–5860.
42. Miller CJ, et al. Rhesus macaques previously infected with simian-/human immunodeficiency virus are protected from vaginal challenge with pathogenic SIV_{mac239}. J Virol 1997;**71**:1911–1921.
43. Musey L, Hu Y, Eckert L, Christensen M, Karchmer T, McElrath MJ. HIV-1 induces cytotoxic T lymphocytes in the cervix of infected women. J Exp Med 1997;**185**:293–303.
44. Butcher EC, Picker LJ. Lymphocyte homing and homeostasis. Science 1996;**272**:60–66.
45. Weinberg AD, English M, Swain SL. Distinct regulation of lymphokine production is found in fresh versus *in vitro* primed murine helper T cells. J Immunol 1990;**144**:1800–1807.
46. Powers GD, Abbas AK, Miller RA. Frequencies of IL-2- and IL-4-secreting T cells in naive and antigen-stimulated lymphocyte populations. J Immunol 1988;**140**:3352–3357.
47. Daynes RA, Araneo BA, Dowell TA, Huang K, Dudley D. Regulation of murine lymphokine production *in vivo*. III. The lymphoid tissue microenvironment exerts regulatory influences over T helper cell function. J Exp Med 1990;**171**:979–96.
48. Hsieh CA, Macatonia SE, Tripp CS, Wolf SF, O'Garra A, Murphy KM. Development of Th1 CD4+ T cells through IL-12 produced by *Listeria*-induced macrophages. Science 1993;**260**:547–549.
49. Trinchieri G. Interleukin-12: A proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. Annu Rev Immunol 1995;**13**:251–276.
50. Snapper CM, Paul WE. Interferon-γ and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. Science 1987;**236**:944–947.
51. Yoshimoto T, Paul WE. CD4+ NK1.1+ T cells promptly produce interleukin 4 in response to *in vivo* challenge with anti-CD3. J Exp Med 1994;**179**:1285–1295.
52. Yoshimoto T, Bendelac A, Watson C, Hu-Li J, Paul WE. Role of NK1.1+ T cells in a Th2 response and in immunoglobulin E production. Science 1995;**270**:1845–1847.
53. Coffman RL, Varkila K, Scott P, Chatelain R. Role of cytokines in the differentiation of CD4+ T-cell subsets *in vivo*. Immunol Rev 1991;**123**:189–207.
54. Mosmann TR. The role of helper T-cell products in mouse B-cell differentiation and isotype regulation. Immunol Rev 1988;**102**:5–28.
55. Mosmann TR, Coffman RL. Th1 and Th2 cells: Different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol 1989;**7**:145–173.
56. Seder RA, Paul WE. Acquisition of lymphokine-producing phenotype by CD4+ T cells. J Allergy Clin Immunol 1994;**94**:1195–1202.
57. Schooley KA, Coffman RL, Mossmann TR, Paul WE. Lymphokine control of *in vivo* immunoglobulin isotype selection. Annu Rev Immunol 1990;**8**:303–333.
58. Esser C, Radbruch A. Immunoglobulin class switching: Molecular and cellular analysis. Annu Rev Immunol 1990;**8**:717–735.

59. Gajewski TF, Fitch FW. Anti-proliferative effect of IFN-γ in immune regulation. I. IFN-γ inhibits the proliferation of Th2 but not Th1 murine helper T-lymphocyte clones. J Immunol 1988;**140**:4245–4252.
60. Czerkinsky C, et al. Detection of human cytokine-secreting cells in distinct anatomical compartments. Immunol Rev 1991;**119**:1–18.
61. Hauer AC, Breese EJ, Walker-Smith JA, MacDonald TT. The frequency of cells secreting interferon γ, IL-4, IL-5 and IL-10 in the blood and duodenal mucosa of children with cow's milk hypersensitivity. Pediatr Res 1997;**42**:1–10.
62. VanCott JL, et al. Regulation of mucosal and systemic antibody responses by T helper cell subsets, macrophages and derived cytokines following oral immunization with live recombinant *Salmonella*. J Immunol 1996;**156**:1504–1514.
63. McGhee JR, Mestecky J, Elson CO, Kiyono H. Regulation of IgA synthesis and immune response by T cells and interleukins. J Clin Immunol 1989;**9**:175–199.
64. Defrance T, Vanbervliet B, Briere F, Durand I, Rousset F, Banchereau J. Interleukin 10 and transforming growth factor b cooperate to induce anti-CD40-activated naive human B cells to secrete immunoglobulin A. J Exp Med 1992;**175**:671–682.
65. Van Vlasselaer P, Punnonen J, de Vries JE. Transforming growth factor-b directs IgA switching in human B cells. J Immunol 1992;**148**:2062–2067
66. Murray PD, McKenzie DT, Swain SL, Kagnoff MF. Interleukin 5 and interleukin 4 produced by Peyer's patch T cells selectively enhance immunoglobulin A expression. J Immunol 1987;**139**:2669–2674
67. Coffman RL, Shrader B, Carty J, Mossman TR, Bond MW. A mouse T-cell product that preferentially enhances IgA production. Biologic characterization. J Immunol 1987;**139**:3685–3690
68. Kiyono H, et al. Murine Peyer's patch T-cell clones: characterization of antigen-specific helper T cells for immunoglobulin A responses. J Exp Med 1982;**156**:1115–1130.
69. Kiyono H, et al. Isotype-specificity of helper T-cell clones: Peyer's patch Th cells preferentially collaborate with mature IgA B cells for IgA responses. J Exp Med 1984;**159**:798–811.
70. Coffman RL, Lebman DA, Shrade B. Transforming growth factor beta specifically enhances IgA production by lipopolysaccharide-stimulated murine B lymphocytes. J Exp Med 1989;**170**:1039–1044.
71. Kim PH, Kagnoff MF. Transforming growth factor-β 1 is a costimulator for IgA production. J Immunol 1990;**144**:3411–3416.
72. Chen Y, Kuchroo VK, Inobe JI, Hafler DA, Weiner HL. Regulatory T-cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. Science 1994;**265**:1237–1240.
73. Weiner HL. Oral tolerance: immune mechanisms and treatment of autoimmune diseases. Immunol Today 1997;**18**:335–343.
74. Khoury SJ, Hancock WW, Weiner HL. Oral tolerance to myelin basic protein and natural recovery from experimental autoimmune encephalomyelitis are associated with down regulation of inflammatory cytokines and differential upregulation of TGF-β, IL-4 and PGE expression in the brain. J Exp Med 1992;**176**:1355–1364.
75. Craig SW, Cebra. JJ Peyer's patches: an enriched source of precursors for IgA-producing immunocytes in the rabbit. J Exp Med 1971;**134**:188–200.
76. Weisz-Carrington P, Roux ME, McWilliams M, Philips-Quagliata JM, Lamm ME. Organ and isotype distribution of plasma cells producing specific antibody after oral immunization: evidence for a generalized secretory immune system. J Immunol 1979;**123**:1705–1708
77. Scicchitano R, Stanisz A, Ernst PB, Bienenstock J. A common mucosal immune system revisited. In: Husband AJ, ed. Migration and homing of lymphoid cells. Boca Raton, FL: CRC Press; 1988. p. 1–34.
78. Nadal D, Albini B, Schlapfer E, Chen C, Brodsky L, Ogra PL. Tissue distribution of mucosal antibody-producing cells specific for respiratory syncytial virus in severe combined immune deficiency (SCID) mice engrafted with human tonsils. Clin Exp Immunol 1991;**3**:358–64.
79. Czerkinsky C, et al. IgA antibody-producing cells in peripheral blood after antigen ingestion:evidence for a common mucosal immune system in humans. Proc Natl Acad Sci USA 1987;**84**:2449–2453.
80. Czerkinsky C, Svennerholm AM, Quiding M, Johnsson R, Holmgren J. Antibody-producing cells in peripheral blood and salivary glands after oral cholera vaccination of humans. Infect Immun 1991;**59**:996–1001.
81. Kantele A. Antibody-secreting cells in the evaluation of the immunogenicity of an oral vaccine. Vaccine 1990;**8**:321–326.
82. Quiding-Järbrink M, et al. Human circulating antigen-specific B cell immunoblasts after mucosal and systemic immunizations: Differential homing commitments and cell surface differentiation markers. Eur J Immunol 1995;**25**:322–327.
83. Quiding-Järbrink M, et al. Differential expression of tissue-specific adhesion molecules on human circulating antibody-forming cells after systemic, enteric and nasal immunizations. A molecular basis for the compartmentalization of effector B-cell responses. J Clin Invest 1997;**99**:1281–1286.
84. Pabst R. Is BALT a major component of the human lung immune system? Immunol Today 1992;**13**:119–122.
85. Kupfer CF, et al. The role of nasopharyngeal lymphoid tissue. Immunol Today 1992;**13**:219–224.
86. Hein WR. Organization of mucosal lymphoid tissue. Curr Top Microbiol Immunol 1999;**236**:1–14.
87. Brandtzaeg P. Immune functions of human nasal mucosa and tonsils in health. In: Bienenstock J, ed. Immunology of the lung and upper respiratory tract. New York: McGraw-Hill; 1984. p. 28–95.
88. Langman JM, Rowland R. The number and distribution of lymphoid follicles in the human large intestine. J Anat 1986;**194**:189–194.
89. O'Leary AD, Swenney EC. Lympho-glandular complexes of the colon: structure and distribution. Histology 1986;**10**:267–283.
90. Crago SS, et al. Distribution of IgA1-, IgA2-, and J chain-containing cells in human tissues. J Immunol 1984;**132**:16–18.
91. Forrest BD, Shearman DJC, La Brooy JT. Specific immune response in humans following rectal delivery of live typhoid vaccine. Vaccine 1990;**8**:209–212.
92. Lehner T, Panagiotidi C, Bergmeier LA, Ping T, Brooks R, Adams SE. A comparison of the immune response following oral, vaginal, or rectal route of immunization with SIV antigens in nonhuman primates. Vaccine Res 1992;**1**:319–330.
93. Lehner T, et al. T- and B-cell functions and epitope expression in nonhuman primates immunized with simian immunodeficiency viral antigen by the rectal route. Proc Natl Acad Sci USA 1993;**90**:8638–8642.
94. Haneberg B, Kendall D, Amerongen HM, Apter FM, Kraehenbuhl JP, Neutra MR. Induction of specific immunoglobulin-A in the small intestine, colon-rectum, and vagina measured by a new method for collection of secretions from local mucosal surfaces. Infect Immun 1994;**62**:15–23.

95. Hopkins S, et al. A recombinant *Salmonella typhimurium* vaccine induces local immunity by four different routes of immunization. Infect Immun 1995;**63**:3279–3286.
96. Moldoveanu Z, Russell MW, Wu HY, Huang W-Q, Compans RW, Mestecky J. Compartmentalization within the common mucosal immune system. Adv Exp Med Biol 1995;**371**:97–102.
97. Nardelli-Haefliger D, et al. Oral and rectal immunization of adult female volunteers with a recombinant attenuated *Salmonella typhi* vaccine strain. Infect Immun 1996;**64**:5219–5224.
98. Hordnes K, Tynning T, Kvam AI, Johnsson R, Haneberg B. Colonization in the rectum and uterine cervix with group B streptococci may induce specific antibody responses in cervical secretions of pregnant women. Infect Immun 1996;**64**:1643–1652.
99. Kozlowski PA, Cu-Uvin S, Neutra MR, Flanigan TP. Comparison of the oral, rectal, and vaginal immunization routes for induction of antibodies in rectal and genital tract secretions of women. Infect Immun 1996;**65**:1387–1394.
100. Eriksson K, et al. Specific-antibody-secreting cells in the rectums and genital tracts of non-human primates following vaccination. Immunol Infect Immun 1998;**66**:5889–5896.
101. Saito H, et al. Generation of intestinal T cells from progenitors residing in gut cryptopatches. Science 1998;**280**:275–278
102. Lubeck MD, et al. Immunogenicity of recombinant adenovirus-human immunodeficiency virus vaccines in chimpanzees following intranasal administration. AIDS Res Hum Retroviruses 1994;**10**:1443–1449.
103. Quiding-Järbrink M, Granström G, Nordström I, Holmgren J, Czerkinsky C. Induction of compartmentalized B-cell responses in human tonsils. Infect Immun 1995;**63**:853–857.
104. Gallichan WS, Rosenthal KL. Specific secretory immune responses in the female genital tract following intranasal immunization with a recombinant adenovirus expressing glycoprotein B of herpes simplex virus. Vaccine 1995;**13**:1589–1595
105. Gallichan WS, Johnson DC, Graham FL, Rosenthal KL. Mucosal immunity and protection after intranasal immunization with recombinant adenovirus expressing herpes simplex glycoprotein B. J Infect Dis 1993;**168**:622–629.
106. Pal S, Peterson EM, de la Maza LM. Intranasal immunization induces long-term protection in mice against a *Chlamydia trachomatis* genital challenge. Infect Immun 1996;**64**:5341–5348.
107. Russell MW, Moldoveanu Z, White PL, Sibert GJ, Mestecky J, Michalek SM. Salivary, nasal, genital, and systemic antibody responses in monkeys immunized intranasally with a bacterial protein antigen and the cholera toxin B subunit. Infect Immun 1996;**64**:1272–1282.
108. Di Tommaso A, et al. Induction of antigen-specific antibodies in vaginal secretions by using a nontoxic mutant of heat-labile enterotoxin as a mucosal adjuvant. Infect Immun 1996;**64**:974–979.
109. Staats HF, Nichols WG, Palker TJ. Systemic and vaginal antibody respones after intranasal immunization with the HIV-1 C4/V3 peptide T1SP10 MN(A). J Immunol 1996;**157**:462–472.
110. Bergquist C, Johansson EL, Lagergard T, Holmgren J, Rudin A. Intranasal vaccination of humans with recombinant cholera toxin B subunit induces systemic and local antibody responses in the upper respiratory tract and the vagina. Infect Immun 1997;**65**:2676–2684.
111. Johansson EL, Rask C, Fredriksson M, Eriksson K, Czerkinsky C, Holmgren J. Antibodies and antibody-secreting cells in the female genital tract after vaginal or intranasal immunization with cholera toxin B subunit or conjugates. Infect Immun 1998;**66**:514–20.
112. Hameleers DM, Stoop AE, van der Ven I, Biewenga J, van der Baan S, Sminia T Intra-epithelial lymphocytes and non-lymphoid cells in the human nasal mucosa. Int Arch Allergy Appl Immunol 1989;**88**:17–22.
113. Graeme-Cook F, Bhan AK, Harris NL. Immunohistochemical characterization of intraepithelial and subepithelial mononuclear cells of the upper airways. Am J Pathol 1993;**143**:1416–22.
114. Kupfer CF, Hameleers DM, Bruijntjes JP, van der Ven I, Biewenga J, Sminia T. Lymphoid and non-lymphoid cells in nasal-associated lymphoid tissue (NALT) in the rat. An immuno- and enzyme-histochemical study. Cell Tissue Res 1990;**259**:371–377.
115. van der Ven I, Sminia T. The development and structure of mouse nasal-associated lymphoid tissue: an immuno- and enzyme-histochemical study. Reg Immunol 1993;**5**:69–75
116. Ogra PL. Effect of tonsillectomy and adenoidectomy on nasopharyngeal antibody response to poliovirus. N Engl J Med 1979;**284**:59–64.
117. Edwards JN, Morris HB. Langerhans'cells and lymphocyte subsets in the female genital tract. Br J Obstet Gynaecol 1985;**92**:974–982.
118. Hussain LA, et al. Expression and gene transcript of Fc receptors for IgG, HLA class II antigens and Langerhans cells in human cervico-vaginal epithelium. Clin Exp Immunol 1992;**90**:530–538.
119. Olaitan A, Johnson MA, MacLean A, Poulter LW. The distribution of immunocompetent cells in the genital tract of HIV-positive women. AIDS 1996;**10**:759–764.
120. Kutteh WH, Hatch KD, Blackwell RE, Mestecky J. Secretory immune system in the female reproductive tract: I. Immunoglobulin and secretory component-containing cells. Obstetr Gynecol 1988;**71**:56–60.
121. Kutteh WJ, Mestecky J. Secretory immunity in the female reproductive tract. Am J Reprod Immunol 1994;**31**:40–46.
122. Ogra PL, Ogra SS. Local antibody response to poliovaccine in the female genital tract. J Immunol 1973;**110**:1307–1311.
123. Wassen L, Schon K, Holmgren J, Jertborn M, Lycke N. Local intravaginal vaccination of the female genital tract. Scand J Immunol 1986;**44**:408–414.
124. Mestecky J, Kutteh WH, Jackson S. Mucosal immunity in the female genital tract: relevance to vaccination efforts against the human immunodeficiency virus. AIDS Res Hum Retroviruses 1994;**10**: S11–S20.
125. Bergman KC, Waldman RH, Tischner H, Pohl W. Antibody in tears, saliva and nasal secretions following oral immunization of humans with inactivated influenza virus vaccine. Int Arch Allergy Appl Immunol 1986;**80**:107–109.
126. Bergman KC, Waldman RH. Oral immunization with influenza virus: Experimental and clinical studies. Curr Top Microbiol Immunol 1989;**146**:83–89.
127. Moldoveanu Z, Clements, ML, Prince SJ, Murphy BR, Mestecky J. Human immune responses to influenza virus vaccines administered by systemic or mucosal routes. Vaccine 1995;**3**:1006–1012.
128. Ohlsson-Wilhelm BM, Duncan JD, Mestecky J, Compans RW. Mucosal immunization against influenza virus using bioadhesive polymers. Proceedings, Options for the Control of Influenza III. 1998. (In press).

129. Gizurarson G, Jonsdottir VM, Heron I. Intranasal administration of diptheria toxoid. Selecting antibody isotypes using formulations having various lipophilic characteristics. Vaccine 1995;13:617–621.
130. Michalek SM, Eldridge JH, Curtiss III R, Rosenthal KL. Antigen delivery systems: new approaches to mucosal immunization. In: Ogra PL, Mestecky J, Lamm ME, StroberW, McGhee JR, Bienenstock J, eds. Handbook of mucosal immunology. San Diego: Academic Press; 1994. p. 373–390.
131. Gould-Fogerite S, et al. Lipid matrix-based subunit vaccines: a structure-function approach to oral and parenteral immunization. AIDS Res Human Retroviruses 1994;10:599–5103.
132. Eldridge JH, Meulbroek JA, Staas JK, Tice TR, Gilley RM. Vaccine containing biodegradable microspheres specifically enter the gut-associated lymphoid tissue following oral administration and induce a disseminated mucosal immune response. Adv Exp Med Biol 1989;251:191–202.
133. Duncan JD, Gilley RM, Schafer DP, Moldoveanu Z, Mestecky J. Poly (lactide-co-glycolide) microencapsulation of vaccines for mucosal immunization. In: Kiyono H, Ogra PL, McGhee JR, eds. Mucosal vaccines. San Diego: Academic Press; 1996. p. 159–173.
134. Mestecky J, Eldridge JH. Targeting and controlled release of antigens for the effective induction of secretory antibody responses. Curr Opin Immunol 1991;3:492–495.
135. Mestecky J, et al. Current options for vaccine delivery systems by mucosal routes. J Controlled Release 1997;48:243–257.
136. McKenzie SJ, Halsey JF. Cholera toxin B subunit as a carrier protein to stimulate a mucosal immune response. J Immunol 1984;133:1818–1824.
137. De Aizpurua HJ, Russell-Jones GJ. Oral vaccination. Identification of classes of proteins that provoke an immune response upon oral feeding. J Exp Med 1988;167:440–451.
138. Czerkinsky C, Russell MW, Lycke N, Lindblad M, Holmgren J. Oral administration of a streptococcal antigen coupled to cholera toxin B subunit evokes strong antibody responses in salivary glands and extramucosal tissues. Infect Immun 1989;57:1072–1077
139. Bergquist C, Lagergard T, Lindblad M, Holmgren J. Local and systemic responses to dextran-cholera toxin B subunit conjugates. Infect Immun 1995;63:2021–2025.
140. Sanchez J, Johansson S, Löwenadler B, Svennerholm A-M, Holmgren J. Recombinant cholera toxin B-subunit and gene fusion proteins for oral vaccination. Res Microbiol 1990;141:971–979.
141. Dertzbaugh MT, Elson CO. Comparative effectiveness of the cholera toxin B subunit and alkaline phosphatase as carriers for oral vaccines. Infect Immun 1993;61:48–55.
142. Sanchez J, Holmgren J. Recombinant system for overexpression of cholera toxin B subunit in *Vibrio cholerae* as a basis for vaccine development. Proc Natl Acad Sci USA 1989;86:481–485.
143. Jobling MG, Holmes RK. Fusion proteins containing the A2 domain of cholera toxin assemble with B polypeptides of cholera toxin to form immunoreactive and functional holotoxin-like chimeras. Infect Immun 1992;60:4915–4924.
144. Hajishengallis G, Hollingshead SK, Koga T, Russell MW. Mucosal immunization with a bacterial protein antigen genetically coupled to cholera toxin A2/B subunits. J Immunol 1995;154:4322–4332.
145. Sultan F, Jin LL, Jobling MG, Holmes RK, Stanley SL Jr. Mucosal immunogenicity of a holotoxin-like molecule containing the serine-rich *Entamoeba histolytica* protein (SREHP) fused to the A2 domain of cholera toxin. Infect Immun 1998;66:462–468.
146. Moxon RE. Applications of molecular microbiology to vaccinology. Lancet 1991;350:1240–1244.
147. Plotkin SA. Vaccination in the 21st century. J Infect Dis 1993;168:29–37.
148. Curtiss R, Kelly SM, Hassan JO. Live oral avirulent *Salmonella* vaccines. Vet Microbiol 1993;37:397–405.
149. Doggett TA, Brown PK. Attenuated *Salmonella* as vectors for oral immunization. In: Kiyono H, Ogra PL, McGhee JR, eds. Mucosal Vaccines. San Diego: Academic Press; 1996. p. 105–108.
150. Chatfield S, Roberts M, Londono P, Cropley I, Douce G, Dougan G. The development of oral vaccines based on live attenuated *Salmonella* strains. FEMS Immunol Med Microbiol 1993;7:1–7.
151. Roberts M, Chatfield, SN, Dougan G. *Salmonella* as carriers of heterologous antigens. In: O'Hagan DT, ed. Novel delivery systems for oral vaccines. Boca Raton, FL: CRC Press; 1994. p. 27–41.
152. Schodel F, Curtiss R III. *Salmonella* as oral vaccine carriers. Dev Biol Stand 1995;84:245–53.
153. Gonzales C, et al. *Salmonella typhi* vaccine strain CVD 908 expressing the circumsporozoite protein of *Plasmodium falciparum*: strain construction and safety about immunogenicity in humans. J Infect Dis 1994;169:927–931.
154. Yang DM, Fairweather N, Button LL, McMaster WR, Kahl LP, Liew FY. Oral *Salmonella typhimurium* (AroA⁻) vaccine expressing a major leishmanial surface protein (gp 63) preferentially induces T helper 1 cells and protective immunity against leishmaniasis. J Immunol 1990;145:2281–2285.
155. Okahashi N, et al. Oral immunization of interleukin-4 (IL-4) knockout mice with a recombinant *Salmonella* strain or cholera toxin reveals that CD4⁺ Th2 cells producing IL-6 and IL-10 are associated with mucosal immunoglobulin A responses. Infect Immun 1996;64:1516–1525.
156. Stover CK. Recombinant vaccine delivery systems and encoded vaccines. Curr Opin Immunol 1994;6:568–571
157. Renauld-Mongénie G, et al. Induction of mucosal immune responses against a heterologous antigen fused to filamentous hemagglutinin after intranasal immunization with recombinant *Bordetella pertussis*. Proc Natl Acad Sci USA 1996;93:7944–7949.
158. Graham FL. Use of human adenovirus-based vectors for antigen expression in animals. J Gen Virol 1989;70:429–434.
159. Wilson JM. Adenoviruses as gene-delivery vehicles. N Engl J Med 1996;334:1185–1187.
160. Graham FL, Smiley J, Russell WC, Nairn R. Characterization of a human cell line transformed by DNA from human adenovirus type 5. J Gen Virol 1977;36:59–74.
161. Gallichan WS, Rosenthal KL. Long-term immunity and protection against herpes simplex virus type 2 in the murine female genital tract after mucosal but not systemic immunization. J Infect Dis 1998;177:1155–1161.
162. Gallichan WS, Rosenthal KL. Long-lived cytotoxic T lymphocyte memory in mucosal tissues after mucosal but not systemic immunization. J Exp Med 1996;184:1879–1890.
163. Buge SL, et al. An adenovirus-simian immunodeficiency virus env vaccine elicits humoral, cellular, and mucosal immune responses in rhesus macaques and decreases viral burden following vaginal challenge. J Virol 1997;71:8531–8541

164. Prevec L, Campbell JB, Christie BS, Belbeck L, Graham FL. A recombinant human adenovirus vaccine against rabies. J Infect Dis 1990;**161**:27–30.
165. Hsu KH, et al. Immunogenicity of recombinant adenovirus-respiratory syncytial virus vaccines with adenovirus types 4, 5 and 7 vectors in dogs and a chimpanzee. J Infect Dis 1992;**166**:769–775.
166. Tacket CO, et al. Initial safety and immunogenicity studies of an oral recombinant adenohepatitis B vaccine. Vaccine 1992;**10**:673–676.
167. Baca-Estrada ME, Liang X, Babiuk LA, Yoo D. Induction of mucosal immunity in cotton rats to haemagglutinin-esterase glycoprotein of bovine coronavirus by recombinant adenovirus. Immunology 1995;**86**:134–140.
168. Kagami H, et al. Repetitive adenovirus administration to the parotid gland: role of immunological barriers and induction of tolerance. Hum Gene Ther 1998;**10**:305–313.
169. Brochier B, et al. Large-scale eradication of rabies using recombinant vaccinia-rabies vaccine. Nature 1991;**354**:520–522.
170. Kieny MP, et al. Expression of rabies virus glycoprotein from a recombinant vaccinia virus. Nature 1984;**312**:163–166.
171. Meitin CA, Bender BS, Small PA Jr. Enteric immunization of mice against influenza with recombinant vaccinia. Proc Natl Acad Sci USA 1994;**91**:11187–11191.
172. Ramsay AJ, Kohonen-Corish M. Interleukin-5 expressed by a recombinant virus vector enhances specific mucosal IgA responses *in vivo*. Eur J Immunol 1993;**23**:3141–3145.
173. Paoletti, E. Applications of poxvirus vectors to vaccination: an update. Proc Natl Acad Sci USA 1996;**93**:11349–11353.
174. Welter J, Taylor J, Tartaglia J, Paoletti E, Stephensen CB. Mucosal vaccination with recombinant poxvirus vaccines protects ferrets against symptomatic CDV infection. Vaccine 1999;17:308–318.
175. Gonin P, Oualikene W, Fournier A, Eloit M. Comparison of the efficacy of replication-defective adenovirus and Nyvac poxvirus as vaccine vectors in mice. Vaccine 1996;**14**:1083–1087.
176. Mayr A, Danner K. Vaccination against pox diseases under immunosuppressive conditions. Dev Biol Stand 1978;**41**:225–234.
177. Sutter G, Wyatt LS, Foley PL, Bennink JR, Moss B. A recombinant vector derived from the host range-restricted and highly attenuated MVA strain of vaccinia virus stimulates protective immunity in mice to influenza virus. Vaccine 1994;**12**:1032–1040.
178. Morrow CD, Novak MJ, Ansardi DC, Porter DC, Moldoveanu Z. Recombinant viruses as vectors for mucosal immunity. Curr Top Microbiol Immunol 1999;**236**:255–273.
179. Caley IJ, et al. Humoral, mucosal, and cellular immunity in response to a human immunodeficiency virus type 1 immunogen expressed by a Venezuelan equine encephalitis virus vaccine vector. J Virol 1997;**71**:3031–3038.
180. Davis NL, Brown KW, Johnston RE. A viral vaccine vector that expresses foreign genes in lymph nodes and protects against mucosal challenge. J Virol 1996;**70**:3781–3787.
181. Pushko P, Parker M, Ludwig GV, Davis NL, Johnston RE, Smith JF. Replicon-helper systems from attenuated Venezuelan equine encephalitis virus: expression of heterologous genes *in vitro* and immunization against heterologous pathogens *in vivo*. Virology 1997;**239**:389–401.
182. Tang S, van Rij R, Silvera D, Andino R. Toward a poliovirus-based simian immunodeficiency virus vaccine: correlation between genetic stability and immunogenicity. J Virol 1997;**71**:7841–7850.
183. Anderson MJ, Porter DC, Moldoveanu Z, Fletcher TM 3rd, McPherson S, Morrow CD. Characterization of the expression and immunogenicity of poliovirus replicons that encode simian immunodeficiency virus SIVmac239 Gag or envelope SU proteins. AIDS Res Hum Retroviruses 1997;**13**:53–62.
184. Wolff JA, et al. Direct gene transfer into mouse muscle *in vivo*. Science 1990;**247**:1465–1468.
185. Ulmer JB, et al. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science 1993;**259**:1745–1749.
186. Yamamoto S, Yamamoto T, Kataoka T, Kuramoto E, Yano O, Tokunaga T. Unique palindromic sequences in synthetic oligonucleotides are required to induce IFN and augment IFN-mediated natural killer activity. J Immunol 1992;**148**:4072–4076.
187. Sato Y, et al. Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. Science 1996;**273**:352–354.
188. Krieg AM, et al. CpG motifs in bacterial DNA trigger direct B-cell activation. Nature 1995;**374**:546–549.
189. Klinman DM, Yi AK, Beaucage SL, Conover J, Krieg AM. CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon γ. Proc Natl Acad Sci USA 1996;**93**:2879–2883.
190. Krieg AM, Love-Homan L, Yi AK, Harty JT. CpG DNA induces sustained IL-12 expression *in vivo* and resistance to *Listeria monocytogenes* challenge. J Immunol 1998;**161**:2428–2434.
191. Jakob T, Walker PS, Krieg AM, Udey MC, Vogel JC. Activation of cutaneous dendritic cells by CpG-containing oligonucleotides: a role for dendritic cells in the augmentation of Th1 responses by immunostimulatory DNA. J Immunol 1998;**161**:3042–3049.
192. Moldoveanu Z, Love-Homan L, Huang WQ, Krieg AM. CpG DNA, a novel immune enhancer for systemic and mucosal immunization with influenza virus. Vaccine 1998;**16**:1216–1224.
193. Fynan EF, Webster RG, Fuller DH, Haynes JR, Santoro JC, Robinson HL. DNA vaccines: protective immunizations by parenteral, mucosal and gene-gun inoculations. Proc Natl Acad Sci USA 1993;**90**:11478–11482.
194. Wang B, et al. Gene inoculation generates immune responses against HIV-1. Proc Natl Acad Sci USA 1993;**90**:4156–4160.
195. Kuklin N, Daheshia M, Karem K, Manickan E, Rouse BT. Induction of mucosal immunity against herpes simplex virus by plasmid DNA immunization. J Virol 1997;**71**:3138–3145.
196. Etchart N, Buckland R, Liu MA, Wild TF, Kaiserlian D. Class I-restricted CTL induction by mucosal immunization with naked DNA encoding measles virus haemagglutinin. J Gen Virol 1997;**78**:1577–1580.
197. Baragazzi ML, et al. Safety and immunogenicity of intramuscular and intravaginal delivery of HIV-1 DNA constructs to infant chimpanzees. J Med Primatol 1997;**26**:27–33.
198. McCluskie MJ, Chu Y, Xia JL, Jessee J, Gebyehu G, Davis HL. Direct gene transfer to the respiratory tract of mice with pure plasmid and lipid-formulated DNA. Antisense Nucleic Acid Drug Dev 1998;**8**:401–414.
199. Okada E, et al. Intranasal immunization of a DNA vaccine with IL-12- and granulocyte-macrophage colony-stimulating factor (GM-CSF)-expressing plasmids in liposomes induces strong mucosal and cell-mediated immune responses against HIV-1 antigens. J Immunol 1997;**159**:3638–3647.

200. Sasaki S, et al. Comparison of intranasal and intramuscular immunization against human immunodeficiency virus type 1 with a DNA-monophosphoryl lipid A adjuvant vaccine. Infect Immun 1998;66:823–826.
201. Klavinskis LS, Barnfield C, Gao L, Parker S. Intranasal immunization with plasmid DNA-lipid complexes elicits mucosal immunity in the female genital and rectal tracts. J Immunol 1999;162:254–262.
202. Jones DH, Clegg JC, Farrar GH. Oral delivery of micro-encapsulated DNA vaccines. Dev Biol Stand 1998;92:149–155.
203. Chen SC, et al. Protective immunity induced by oral immunization with a rotavirus DNA vaccine encapsulated in microparticles. J Virol 1998;72:5757–5761.
204. Darji A, et al. Oral somatic transgene vaccination using attenuated *S. typhimurium*. Cell 1997;91:765–775.
205. Paglia P, Medina E, Arioli I, Guzmán CA, Colombo MP. Gene transfer in dendritic cells, induced by oral DNA vaccination with *Salmonella typhimurium*, results in protective immunity against a murine fibrosarcoma. Blood 1998;92:3172–3176.
206. Condon C, Watkins SC, Celluzi CM, Thompson K, Falo LD Jr. DNA-based immunization by *in vivo* transfection of dendritic cells. Nat Med 1996;2:1122–1128.
207. Casares S, Inaba K, Brumeanu TD, Steinman RM, Bona C. Antigen presentation by dendritic cells after immunization with DNA encoding a major histocompatibility complex class II-restricted viral epitope. J Exp Med 1997;186:1481–1486.
208. Chattergoon MA, Robinson TM, Boyer JD, Weiner DB. Specific immune induction following DNA-based immunization through *in vivo* transfection and activation of macrophages. J Immunol 1998;160:5707–5718.
209. Akbari O, Panjwani N, Garcia S, Tascon R, Lowrie D, Stockinger B. DNA vaccination: transfection and activation of dendritic cells as key events for immunity. J Exp Med 1999;189:169–177.
210. Mason HS, Lam DMK, Arntzen CJ. Expression of hepatitis B surface antigen in transgenic plants. Proc Natl Acad Sci USA 1992;89:11745–11749.
211. Arntzen CJ, Mason HS. Oral vaccine production in the edible tissues of transgenic plants. In: Levine MM, Woodrow GC, Kaper JB, Cobon GS, eds. New generation vaccines. New York: Marcel Dekker; 1996. p. 263-267.
212. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998;392:245–252.
213. Mayordomo JI, et al. Bone marrow-derived dendritic cells serve as potent adjuvants for peptide-based antitumor vaccines. Stem Cells 1997;15:94–63.
214. Timmerman JM, Levy R. Dendritic cell vaccines for cancer immunotherapy. Annu Rev Med 1999;50:507–529.
215. Bender A, Bui LK, Feldman MAV, Larson M, Bhardwaj N. Inactivated influenza virus, when presented on dendritic cells, elicits human $CD8^+$ cytolytic T-cell responses. J Exp Med 1995;182:1663–1671.
216. Mbow ML, Zeidner N, Panella N, Titus RG, Piesman J. *Borrelia burgdorferi*-pulsed dendritic cells induce protective immune response against tick transmitted spirochetes. Infect Immun 1997;65:3386–3390.
217. Brossart P, Goldrath AW, Butz EA, Martin S, Bevan MJ. Virus-mediated delivery of antigenic epitopes into dendritic cells as a means to induce CTL. J Immunol 1997;158:3270–3276.
218. Su H, Messer R, Whitmire W, Fischer E, Portis JC, Caldwell HD. Vaccination against chlamydial genital tract infection after immunisation with dendritic cells pulsed with nonviable chlamydiae. J Exp Med 1998;188:809–818.
219. Spangler BD. Structure and function of cholera toxin and the related *Escherichia coli* heat-labile enterotoxin. Microbiol Rev 1992;56:622–647.
220. Fukuta S, Magnani JL, Twiddy EM, Holmes RK, Ginsburg V. Comparison of the carbohydrate-binding specificities of cholera toxin and *Escherichia coli* heat-labile enterotoxins LTh-I, LT-IIa, and LT-IIb. Infect Immun 1988;56:1748–1753.
221. Elson CO, Ealding W. Cholera toxin did not induce oral tolerance in mice and abrogated oral tolerance to an unrelated antigen. J Immunol 1984;133:2892–2898.
222. Lycke N, Holmgren J. Strong adjuvant properties of cholera toxin on gut mucosal immune responses to orally presented antigens. Immunology 1986;2:301–308.
223. Clements JD, Hartzog NM, Lyon FL. Adjuvant activity of *Escherichia coli* heat-labile enterotoxin and effect on the induction of oral tolerance in mice to unrelated protein antigens. Vaccine 1988;6:269–277.
224. Xu-Amano J, et al. Helper T-cell subsets for immunoglobulin A responses: oral immunization with tetanus toxoid and cholera toxin as adjuvant selectively induces Th2 cells in mucosa associated tissues. J Exp Med 1990;172:95–103.
225. Munoz E, Zubiaga AM, Merrow M, Sauter NP, Huber BT. Cholera toxin discriminates between T helper 1 and 2 cells in T cell receptor-mediated activation: role of cAMP in T cell proliferation. J Exp Med 1992;175:131–138.
226. Marinaro M, et al. Mucosal adjuvant effect of cholera toxin in mice results from induction of T helper 2 (Th2) cells and IL-4. J Immunol 1995;155:4621–4629.
227. Snider DP, Marshall, JS, Perdue MH, Liang H. Production of IgE antibody and allergic sensitization of intestinal and peripheral tissues after oral immunization with protein Ag and cholera toxin. J Immunol 1994;153:647–657.
228. Wilson AD, Bailey M, Williams NA, Stokes CR. The *in vitro* production of cytokines by mucosal lymphocytes immunized by oral administration of key-hole limpet hemocyanin using cholera toxin as an adjuvant. Eur J Immunol 1991;21:2333–2339.
229. Sun JB, Holmgren J, Czerkinsky C. Cholera toxin B subunit: an effective transmucosal carrier delivery system for induction of peripheral immunological tolerance. Proc Natl Acad Sci USA 1994;91:10795–10799.
230. Bowen JC, Nair SK, Reddy R, Rouse BT. Cholera toxin acts as a potent adjuvant for the induction of cytotoxic T-lymphocyte responses with non-replicating antigens. Immunology 1994;81:338–342.
231. Lycke N, Tsuji T, Holmgren J. The adjuvant effect of *Vibrio cholerae* and *Escherichia coli* heat-labile enterotoxins is linked to their ADP-ribosyltransferase activity. Eur J Immunol 1992;22:2277–2281.
232. Takahashi I, et al. Mechanisms for mucosal immunogenicity and adjuvancy of *Escherichia coli* labile enterotoxin. J Infect Dis 1996;173:627–635.
233. Douce G, et al. Mutants of *Escherichia coli* heat-labile toxin lacking ADP-ribosyltransferase activity act as nontoxic, mucosal adjuvants. Proc Natl Acad Sci USA 1995;92:1644–1648.
234. Dickinson BL, Clements JD. Dissociation of *Escherichia coli* heat-labile enterotoxin adjuvanticity from ADP-ribosyltransferase activity. Infect Immun 1995;63:1617–1623.
235. Yamamoto S, et al. A nontoxic mutant of cholera toxin elicits Th2-type responses for enhanced mucosal immunity. Proc Natl Acad Sci USA 1997;94:5267–5272.
236. Yamamoto S, et al. Mutants in the ADP-ribosyltransferase cleft of cholera toxin lack diarrhoeagenicity but retain adjuvanticity. J Exp Med 1997;185:1203–1210.

237. Challacombe SJ, Tomasi TB Jr. Systemic tolerance and secretory immunity after oral immunization.
J Exp Med 1980;**152**:1459–1472.
238. Karpus WJ, Lukacs NW. The role of chemokines in oral tolerance: abrogation of nonresponsiveness by treatment with anti-MCP-1.
Ann N Y Acad Sci 1996;**778**:133–142.
239. Strobel S, Mowat AM, Drummond HE, Pickering MG, Ferguson A. Immunological responses to fed protein antigens in mice. II Oral tolerance for CMI is due to activation of cyclophosphamide-sensitive cells by gut-processed antigen.
Immunology 1983;**49**:451–456.
240. Bruce MG, Strobel, S, Hanson DG, Ferguson A. Transferable tolerance for cell-mediated immunity after feeding is prevented by radiation damage and restored by immune reconstitution.
Clin Exp Immunol 1987;**70**:611–618.
241. Mayer L, So LP, Yio XY, Small G. Antigen trafficking in the intestine.
Ann N Y Acad Sci 1996;**778**:28–35.
242. Kaiserlian D. The intestinal epithelial cell: a non conventional type of antigen-presenting cell. In: Auricchio S, Ferguson A, Troncone R, eds. Mucosal immunity and the gut epithelium: interactions in health and disease. Basel: Karger; 1995. p. 32–39.
243. Eynon EE, Parker DC. Small B cells as antigen-presenting cells in the induction of tolerance to soluble protein antigens
J Exp Med 1992;**175**:131–138.
244. Fuchs EJ, Matzinger P. B cells turn off virgin but not memory T cells.
Science 1992;**258**:1156–1159.
245. MacPhersonGG, Liu LM. Dendritic cells and Langerhans cells in the uptake of mucosal antigens.
Curr Top Microbiol Immunol 1999;**236**:34–53.
246. Stumbles PA, et al. Resting respiratory tract dendritic cells preferentially stimulate T helper cell type 2 responses and require obligatory cytokine signals for induction of Th1 immunity.
J Exp Med 1998;**188**:2019–2031.
247. Steptoe RJ, Thomson AW. Dendritic cells and tolerance induction.
Clin Exp Immunol 1996;**105**:397–402.
248. Viney JL, Mowat AM, O'Malley JM, Williamson E, Fanger NA. Expanding dendritic cells in vivo enhances the induction of oral tolerance.
J Immunol 1998;**160**:5815–5825.
249. Khoury SJ, Lider O, Al-Sabbagh A, Weiner HL. Suppression of experimental autoimmune encephalomyelitis by oral administration of myelin basic protein. III. Synergistic effect of lipopolysaccharide.
Cell Immunol 1982;**131**:302–310.
250. Rebien W, Puttonen E, Maasch HJ, Stix E, Wahn U. Clinical and immunological response to oral and subcutaneous immunotherapy with grass pollen extracts. A prospective study.
Eur J Pediatry 1982;**138**:341–344.
251. Wormann F. Oral hyposensitization of children with pollinosis or house dust asthma.
Allergol Immunopathol 1977;**5**:15–26.
252. Hoyne GF, O'Hehir RE, DC Wraith, Thomas WR, Lamb JR. Inhibition of T cell and antibody responses to house dust mite allergen by inhalation of the dominant T cell epitope in naive and sensitized mice.
J Exp Med 1993;**178**:1783–1788.
253. Hanson DG, Vaz NM, Rawlings LA, Lynch JM. Inhibition of specific immune responses by feeding protein antigens. II. Effects of prior passive and active immunization.
J Immunol 1979;**122**:2261–2266.
254. Staines NA, Harper N, Ward FJ, Thompson HSG, Bansal S. Arthritis: animal models of oral tolerance.
Ann N Y Acad Sci 1996;**778**:297–305.
255. Czerkinsky C, et al. Cholera toxin B subunit as transmucosal carrier-delivery and immunomodulating system for induction of anti-infectious and anti-pathological immunity.
Ann N Y Acad Sci 1996;**778**:185–193.
256. Sun JB, Rask C, Olsson T, Holmgren J, Czerkinsky C. Treatment of experimental autoimmune encephalomyelitis by feeding myelin basic protein conjugated to cholera toxin B subunit.
Proc Natl Acad Sci USA 1996;**93**:7196–7201.
257. Tarkowski A, Sun JB, Holmdahl R, Holmgren J, Czerkinsky C. Treatment of experimental autoimmune arthritis by nasal administration of a type II collagen-cholera toxoid conjugate vaccine.
Arthritis Rheum (In press).
258. Bergerot I, et al. A cholera toxoid-insulin conjugate as oral vaccine against spontaneous autoimmune diabetes.
Proc Natl Acad Sci USA 1997;**94**:4610–4614.
259. Ma D, Mellon J, Niederkorn JY. Conditions affecting enhanced corneal allograft survival by oral immunization.
Invest Ophthalmol Vis Sci 1998;**39**:1835–1846.
260. Tamura S, Hatori E, Tsuruhara T, Aizawa C, Kurata T. Suppression of delayed-type hypersensitivity and IgE antibody responses to ovalbumin by intranasal administration of *Escherichia coli* heat-labile enterotoxin B subunit-conjugated ovalbumin.
Vaccine 1997;**15**:225–229.
261. Julia V, Rassoulzadegan M, Glaichenhaus. Resistance to *Leishmania major* induced by tolerance to a single antigen.
Science 1996;**274**:421–423.
262. McSorley SJ, Rask C, Pichot R, Julia V, Czerkinsky C, Glaichenhaus, N. Selective tolerization of Th1-like cells after nasal administration of a cholera toxoid-LACK conjugate.
Eur J Immunol 1998;**28**:44–430.
263. Sun JB, et al. Intranasal administration of a *Schistosoma mansoni* glutathione S-transferase-cholera toxoide conjugate vaccine epokes antiparasitic and antipathological immunity in mice.
J Immunol 1999;**163**:1045–1052.
264. Arakawa T, Yu J, Chong DK, Hough J, Engen PC, Langridge WH. A plant-based cholera toxin B subunit-insulin fusion protein protects against the development of autoimmune diabetes.
Nat Biotechnol 1998;**16**:934–938.
265. Roy K, Mao HQ, Huang SK, Leong KW. Oral gene delivery with chitosan-DNA nanoparticles generates immunologic protection in a murine model of peanut allergy.
Nat Med 1999;**5**:387–391.